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Table of Contents

	Page	
	Original	Translation
Editor's Note		i
Diskalenko, A. P. Preface	3	iii
Table of Contents	164	v
Ginsburg, N. N. Further Development of the Knowledge on Anthrax	4	1
Rudnev, G. P. Anthrax in Man	22	27
Anthraxin and the Reactions Caused by It		
Shliakhov, E. N. Immuno-Biological Investigations with Preparations of the Anthrax Allergen--Anthraxin	29	40
Ivanov, M. I. Experiences on the Immunological Diagnosis of Anthrax	81	113
Zubairov, Z. S. and Timofeev, M. K. Investigation of Anthraxin for the Diagnosis of Anthrax and the Determination of the Post-Infectious and Post-Vaccinal Immuno-Allergic Reactivity	87	120
Senchuk, T. T. Some Results of Anthraxin Tests in Persons Immunized with the STI Vaccine and in Patients	92	127
Mnatsakanian, A. G. Anthraxin Tests in Patients and Past Sufferers from Anthrax	95	131

Table of Contents

	Page	
	Original	Translation
Shliakhov, E. N. and Shvarts, S. A. Chemical Anthraxin - Some Biological, Biochemical and Immuno-Chemical Data	97	133
Shliakhov, E. N. and Shroit, I. G. Patho-Morphological Changes in the Skin Caused Through Tests with Anthraxin	117	164
Arkhipova, V. R. Production of a Standard Live Anthrax Vaccine	121	173
Methods of Laboratory Diagnosis and Identification of the Causative Organisms		
Gruz, E. V. Diagnostic Value of Some Morphological, Cultural and Biochemical Characteristics of the Anthrax Bacillus	124	177
Shliakhov, E. N. and Gruz, E. V. Accelerated Identification of Pathogenic Anthrax Bacilli with the Aid of the Biological Test for Incapsulation	139	198
TSokolenko, D. T. and Chernyi, P. B. Identification and Determination of the Pathogenic Properties of the Anthrax Bacillus with the Aid of the Biological Test for Incapsulation	150	213
Gruz, E. V. Tests with the Specific Bacteriophage "Ba-9" for the Identification of Anthrax Bacilli	152	216
Shliakhov, E. N., Shvarts, S. A. and Burdenko, T. A. Diffusive Gel Precipitation as a Method for the Detection of the Anthrax Antigen. (~~Preliminary~~ Communication)	157	223

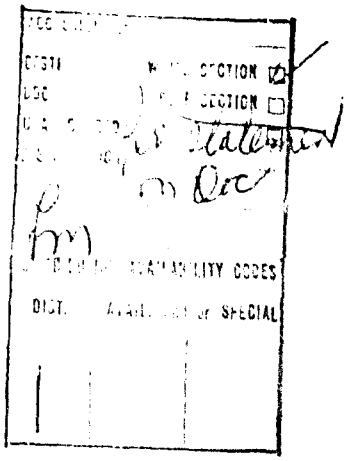

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Table 11

Determination of Desoxyribonucleic Acid and Ribonucleic Acid

Kind of Preparation		Desoxyribonucleic Acid	Ribonucleic Acid
Chemical anthraxin:	1st series	1.9	39.1
	4th series	2.2	46.8
	6th series	1.7	39.6
	8th series	1.9	31.5
	9th series	1.1	37.2
	11th series	1.1	45.0
	12th series	2.0	47.0
	18th series	2.9	30.3
Preparation	No. 19	0.5	11.5
	No. 21	1.3	13.6
	No. 22	0.6	17.4
	No. 23	0	4.5
	No. 24 a	0.6	6.0
	No. 24 b	0	15.0
	No. 24 c	1.7	86.4
	No. 25	0.9	19.0
	No. 26	1.6	39.8

In all cases in which precipitating anthrax serum and preparations of the chemical anthraxin or other preparations under test were used, we observed the formation of one precipitation line (see Drawing 4 in original).

In Table 12 are shown the final antigen dilutions still producing positive reactions (titer of the reactions).

For a more detailed analysis a number of the preparations was subjected to a further treatment and the gel precipitation tests were repeated.

After treatment of the anthraxin with trichloracetic acid the centrifugate, after neutralization or dialysis, fully kept its precipitating properties whereas the sediment (after a corresponding treatment) did not react in precipitation tests.

Table 12

Gel Precipitation Tests

Kind of Preparation		Titer of Reaction	Kind of Preparation	Titer of Reaction
Experimental Chemical Anthraxin:	5th series	120	Preparation No. 13	820
	7th series	160	No. 14	800
	8th series	140	No. 19	100
	9th series	100	No. 21	20
	10th series	110	No. 22	1
	11th series	110	No. 23	160
	18th series	120	No. 24 a	60
Routinely produced Chemical Anthraxin	1st series	140	No. 24 b	20
	2nd series	140	No. 24 c	10
	3rd series	150	No. 25	90
	4th series	160	No. 26	120
	5th series	170		
	6th series	140		
	7th series	100		
	8th series	100		
	9th series	110		

Anthraxin subjected to tryptic digestion remained capable of reacting with anthrax precipitating serum while anthraxin subjected for 3 hours to hydrolysis with a half-normal solution of sulfuric acid completely lost its precipitating properties.

After the salting-out of the proteins from the chemical anthraxin with the aid of ammonium sulfate the preparations continued to react in precipitation tests, whereas solutions of the salted-out proteins failed to do so.

We attempted to obtain gel precipitation reactions not only with precipitating serum but also with various series of therapeutic anthrax sera and anthrax-globulin for medicinal use. However, it was not possible to obtain precipitation reactions with the two last mentioned products owing to their considerably reduced contents in precipitins.

The removal of the easily extractable lipids from the chemical anthraxin (series 25 and 26) did not influence the intensity of the precipitation reactions.

No positive results were obtained in gel precipitation tests with the blood of hyperimmune guinea pigs in which skin-allergic tests with anthraxin had proved positive.

Electrophoretic and Immuno-Electrophoretic Investigations

A series of attempts to investigate the protein composition of the chemical anthraxin with the aid of electrophoresis did not give positive results. The small amounts of protein present in the preparation even after its concentration could not be observed with the aid of paper electrophoresis.

Immuno-electrophoretic investigations were made with the aid of a half-micromodification of the method of P. Grabar (37) in an altered chamber constructed in our laboratory.

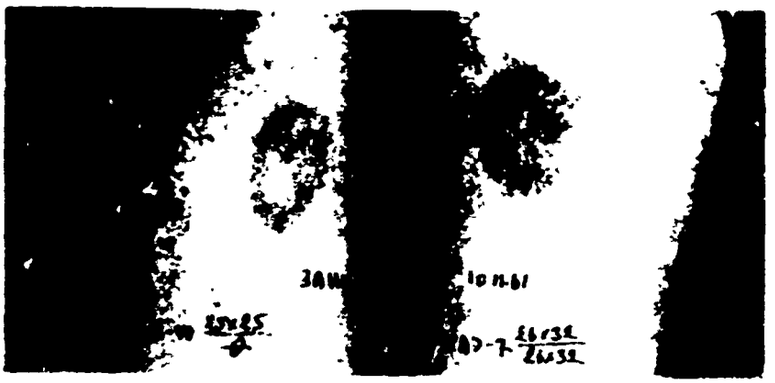
Electrophoretic tests were made simultaneously in two agar dishes, one of which was subjected to staining and served as control.

In part of the tests we subjected chemical anthraxin to electrophoresis in agar, followed by the introduction of standard anthrax precipitating serum into the lateral trench. After one day one could note the presence of one arc of precipitation, the position of which in relation to the site of placement of the preparation depended solely upon the length and intensity of the electrophoresis. The presence of separate fractions in the preparation could not be observed.

In other tests we subjected the anthrax precipitating serum to the electrophoretic dispersal and put the chemical anthraxin into the lateral trench. In these cases we could note the presence of a precipitation line in front of the site of the gamma-globulin fraction of the serum protein.

Evaluation of the Results and Conclusions

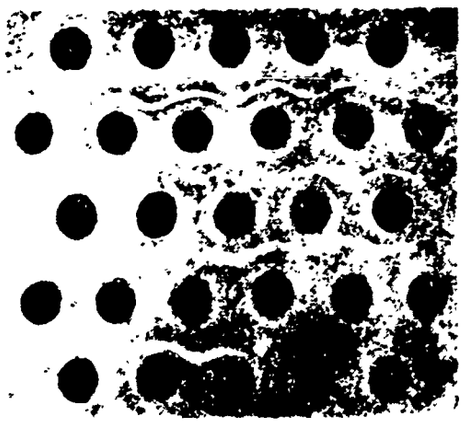
The results of the tests on immunized (or hyperimmunized) guinea pigs indicate that all investigated series of the chemical anthraxin produce skin-allergic reactions, exceeding in general the level conditionally accepted for this preparate. At the same time not immune animals do not show skin-allergic reactions.



Drawing 1. G. Z. vaccinated subcutaneously 15 days before performance of the skin-allergic reaction. 24 hours after the administration of chemical anthraxin a +++ reaction appeared on the left forearm. On the right forearm after administration of tissue anthraxin a + reaction appeared.

Drawing 2. Chromatogram of the amino-acid composition of chemical anthraxin (in the center). To the left and right evidence of (1) cystine; (2) lysine; (3) arginine; (4) aspariginic acid; (5) glycocoll; (6) glutamic acid; (7) threonine; (8) proline; (9) thyrosine; (10) valine; (11) phenylalanine; (12) leucine.

Drawing 3. Chromatogram of the carbohydrate composition of the chemical anthraxin. (1) Glucosamine; (2) Galactose; (3) Ribose



Drawing 4. Diffusive precipitation in agar gel. 2nd and 4th rows of holes - precipitating anthrax serum Remaining holes - various dilutions of routinely produced chemical anthraxin Series 1. 1st row dilutions of 1, 10, 20, 30, 40; 3rd row dilutions of 50, 60, 70, 80, 90; 5th row dilutions of 100, 120, 140, 160, 180.

On the other hand the preparates made from the polysaccharides of the anthrax bacillus (Nos.19, 21, 23 and 24) respond to intracutaneous administration with a local reaction below the conditional level which in the majority of cases is slightly distinquishable from the reaction obtained in the control animals.

The preparations of the chemical anthraxin Nos.25 and 26, from which the easily extractable lipids had been removed, produced an allergic reaction in the immunized animals but failed to do so in the controls, i.e. they did not differ from the standard chemical anthraxin in their action.

The protein fraction, obtained in the process of isolation of the polysaccharides from the anthrax bacilli (preparate No. 22) produced a local inflammatory reaction with necrosis in the immunized animals and also a considerable, though somewhat slighter reaction in the control animals.

The concentrated preparations of the chemical anthraxin (No. 14 and No. 17) produced a reaction similar to that obtained with the standard preparation. In our opinion this phenomenon is related to the level of the patho-physiological reactivity of the animal body which has a definite limit.

Tests comparing the action of the chemical and tissue anthraxin in immunized persons indicated in general a higher biological activity of the first mentioned preparate. It was also found that after simultaneous administration of both preparates to one and the same person the fixation of the chemical anthraxin by the organism takes place more intensively, while at the same time the level of the allergic reaction to the tissue vaccine is decreased. The separate administration of the preparates to persons with an established post-vaccinal immunity (e.g. on the 112th day after vaccination) produces identical results of the positive reactions, while during the period of formation of the immunity the percentage of persons positively reacting to the chemical anthraxin is higher than when the tissue anthraxin is used.

Similar results were obtained when using either preparation in anthrax patients.

The observation of an "Aufflammung" of the skin-allergic reactions at the site of the previous administration of the chemical anthraxin could serve as a criterium for the time of onset of the immuno-allergic transformation of the organism.

The absence of sensitizing properties of the chemical anthraxin following its repeated administration to man has been observed.

Biochemical investigations of the chemical anthraxin showed that the average conterts of the dry residue (without taking the introduced mineral substances into account) equal 0.25% and that the mineral substances form about 20% of the dry residue. The contents of the standard preparations in total nitrogen vary from 13.5 to 23.3 mg %; the residual nitrogen within a range from 13 to 17.8 mg %.

The reducing substances accounted for in mg % of glucose, varied from 5.8 to 9.8; after energetic hydrolysis with mineral acids, as a result of the splitting of the polysaccharides, from 13.4 to 20.1 mg %. In this case the total amount of amino-sugars varies within the range of 2.8-4.9 mg % and the amount of hexosamines from 0.75 to 1.4 mg %.

One could extract from the chemical anthraxin about 36 mg % of lipids and after energetic acid hydrolysis an additional amount of 12 mg %. The contents of the standard chemical anthraxin in total phosphorus varied from 2.6 to 5.8 mg %. Through repeated tests with chemical anthraxin one could establish the presence of from 1.1 to 2.9 mg % of desoxyribonucleic acid and of 30-47 mg % of ribonucleic acid.

In contrast to the standard chemical anthraxin one observed in the polysaccharide preparations of the anthrax bacillus a considerably reduced amount of total nitrogen (2-7.2 mg %) and an absence of residual nitrogen; an absence or traces of phosphorus; an absence or traces of desoxyribonucleic acid and inconsiderable amounts of ribonucleic acid (except in Barber's preparation "c" in which a larger amount of the ribonucleic acid was found than in the chemical anthraxin).

All preparations of the chemical anthraxin showed in agar gel precipitation tests with horse precipitating anthrax serum one distinct line of precipitation in dilutions of the anthraxin of 1:100 to 1:170. The polysaccharide preparations also gave one line precipitation most active in this respect was the preparate from the polysaccharide of Ivanovics (in a dilution of up to 1:160), least active the preparate "c" of Barber (up to a dilution of 1:10). The "protein" preparate (No. 22) gave a precipitation line only when used undiluted. The removal of the easily extractable lipids from the chemical anthraxin exerted no substantial influence on the titer of the precipitation reactions (1:90-1:120).

The tryptic digestion of the chemical anthraxin or the removal of the proteins with the aid of trichloracetic acid exerted no influence on the character and titer of the precipitation reactions, while an energetic acid hydrolysis fully suppressed the reactions.

A comparison of the biochemical and immuno-chemical characteristics of the chemical anthraxin and of the other preparations with their biological activity permits to arrive at some conclusions.

A complete biological activity, i.e. the ability of producing the conditional (or more marked) skin-allergic reactions within the optimal periods after the immunization of guinea pigs and its absence in the control animals was shown by the concentrated (native) chemical anthraxin and by this preparate after the removal of the easily extractable lipids.

Thus the presence of a considerable amount of lipids in the preparate does not endow it with unspecific or necrotizing properties, as has been found in the case of the tularemia allergen (38, 39).

Taken by itself the polysaccharide component of the chemical anthraxin stands on account of its composition (presence of hexosamine and galactose) nearer to the polysaccharides of Ivanovics (12), "c" of Staub and Graber (16) and "a" and "c" isolated by Barber (17, 18) but at the same time it contains ribose which takes part in the composition of another polysaccharide isolated by a number of authors.

While, as far as the property of becoming precipitated by the precipitating horse anthrax serum is concerned, the polysaccharide of Ivanovics ranks equally with the standard chemical anthraxin and the other polysaccharides react less actively in this respect, they are all below par in respect to their biological activity.

To judge from the chromatographic test, the chemical anthraxin contains 10 amino-acids. It differs from the "polysaccharides" allergen of P. pestis, studied by Bakhrakh, Egorova and Pavlova (40) by the presence of arginine and phenyl-alanine and the absence of cystine, alanine and proline.

Immuno-chemical investigations permitted to establish that the chemical anthraxin becomes precipitated by the gamma-globulin fraction of the anthrax precipitating serum; this is in agreement with the laws governing the precipitation of the polysaccharides of B. anthracis noted by Grabar and Staub (25, 26).

Speaking of the allergens of the different microbial species, in contrast to the assertions of some authors (41), it is not permissible to expect some common features of the chemical structure characterizing the specific properties of any allergen. A number of authors, studying this problem, established the protein nature of the dysentery allergen (41), the lipid-protein (42) or lipid-polysaccharide (43) nature of the tuberculin, the presence of a polypeptide-polysaccharide complex in the P. pestis allergen (40), of a water-soluble protein fraction in lepromin (44), etc.

Our investigations permit to assume that the biologically fully active chemical anthraxin consists of a protein-polysaccharide-nuclein complex. In respect to its general biochemical character the chemical anthraxin stands near to the second fraction isolated from virulent tularemia bacilli by Shipitsina (38) which is endowed with a marked allergenic function.

It is possible that in the complex characterizing the chemical anthraxin a definite role of biological activity is played by the A-lipoid fraction remaining in the preparate after the removal of the easily extractable lipids.

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(#93)

PATHOMORPHOLOGICAL CHANGES IN THE SKIN CAUSED THROUGH TESTS WITH ANTHRAXIN

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(Original pp. 117-120)

The performance of cutaneous allergic tests (with tuberculin, tularin, brucellin, mallein, etc.) represents a specific method for the detection of the immuno-allergic reactivity of the macroorganism to the respective antigen. Studies of these tests in various directions, including morphological investigations are not only of practical but also of theoretical importance, inasmuch as they help to reveal the laws underlying the formation and development of the local pathological processes as well as of the morbid process in general.

Thus far the patho-morphology of the skin lesions resulting from allergic tests with anthraxin has not been studied. We (Shliakhov, 1958) made clinical observations on the dependence of this skin reaction in animals upon various factors. In the present communication we report some patho-histological findings regarding the character of the local morbid processes in relation to the quantity of the allergen administered to immunologically reactive (specifically sensitized) experimental animals.

For this purpose we made 3 series of observations in 30 guinea pigs weighing 450-500 g.

1st Series. Three days before the tests two symetrically situated areas (with a diameter of 3 cm each) on the flanks of 12 normal (not immunized) guinea pigs were freed from hairs. Into the skin on the left of these areas 0.12-0.15 ml of tissue anthraxin were injected, while into the skin of the right of the areas the same amount of a control fluid (vehicle of the anthraxin) was administered. Pairs of the animals were sacrificed 3, 6, 12, 24, 48 and 72 hours after these injections had been made.

2nd Series. The same method was used for 12 guinea pigs which 60-98 days before the test had been immunized with the STI vaccine for animals. The animals were sacrificed as the case of Series 1.

3rd Series. 6 guinea pigs which had been immunized 15 days previously with STI vaccine were injected on the left side with 0.2-0.3 ml of anthraxin and on the right side with the same amount of the control fluid. All animals were sacrificed after 24 hours.

After the animals had been killed, the skin of the injected parts together with the subcutaneous layer and the muscles under it were cut out; the specimens were hardened in a 10% solution of neutral formol and serial sections were stained in the usual manner.

Results of the Patho-Histological Studies

Skin Lesions Resulting from the Injection of the Allergen and the Test Control Fluid into Not Immune Guinea Pigs (1st Series)

At the site of administration of the anthraxin there forms a small focus of inflammation. Already at the 6th hour after the test one finds a not well defined infiltrate consisting of neutrophile leucocytes and rare lymphocytes surrounded by a zone of moderate edema and hyperemia with perivascular agglomerations of lymphocytes. Small infiltrates are found also in the lower part of the loose tissue adjacent to the muscular layer.

24 hours after the beginning of the test the cell infiltrate becomes somewhat more considerable (see Drawing 1 in original). The epithelial cells near the focus show marked signs of dystrophia (swelling, vacuolization of the cytoplasma and pycnosis of the nuclei) and at some places are infiltrated with leucocytes.

After 48 hours and particularly after 72 hours only small cell infiltrates remain in the upper layer of the dermis, surrounded by a narrow zone of edema.

At the site of administration of the control fluid, in comparison with those described above, the changes are quite insignificant. They consist of a slight edema of the dermis and irritation of the elements of the local connective tissue. After 24 hours a small infiltrate forms, consisting of lymphogenous and histiocytic elements (see Drawing 2 in original). Later on these alternations regress rapidly.

Skin Changes after the Administration of a Moderate Dose of Anthraxin (and of the Control Fluid) to Immunized Guinea Pigs (2nd Series)

At the site of administration of the control fluid one notes changes analogous to those described above. At the site of anthraxin administration there develops, as in the animals of the first series, a process of inflammation which, however, differs in its degree and in morphological details.

The process of inflammation is more extensive, involving a larger area, and accompanied by a quite marked edema and hyperemia and considerable perivascular infiltrations of mononuclear cells. These processes reach their maximum 24 hours after the beginning of the tests.

It is possible to distinguish already macroscopically the zone of cutaneous hyperemia and thickening of the skin layer exceeding the norm 2.-2.5 times. Microscopically one observes at the site of administration of the anthraxin small hemorrhages in the loosened epidermis which is infiltrated with round cells. The dermis of this area is infiltrated with leucocytes (see Drawing 3 in original). The cellular infiltrate is surrounded by a wide zone of hyperemic vessels; in many of them one can note agglomerations of cells, among which lymphocytes and histiocytes are prominent. The majority of the capillaries is enlarged and filled with blood, their endothelia are swollen. The signs of inflammation are not marked at the periphery of the zone of hyperemia and edema.

Thus schematically the changes in the dermis appear in the form of three concentric zones: (a) a dense cell infiltrate at the site of the anthraxin administration (consisting of disintegrating neutrophiles and mainly of lymphoid and histiocytary elements); (b) a zone of hyperemia and perivascular inflammatory infiltrates and (c) a zone of slight edema with sparse agglomerations of cellular elements.

In the loose tissue layer the inflammatory processes are considerably more extensive than in the zone of hyperemia and infiltration ofthe dermis (see Drawing 4 in original). Characteristic is that perivascular cell agglomerations are present not only in the border zone between the loose tissue layer and the musculature but in some places extend into the latter, accompanied by edema and hyperemia.

After 48 hours and specially after 72 hours the inflammatory processes regress, mainly through a decrease of the exudative manifestations. There remain a small focus of inflammation and single leucocytic infiltrates in the dermis (see Drawing 5 in original). from which in some places perivascular infiltrates descend (see Drawing 6 in original). Single foci of inflammation are noted in the tissue layer bordering on the musculature.

Changes in the Skin after the Administration of an Increased Anthraxin Dose to Immunized Guinea Pigs (3rd Series of Tests)

After 24 hours there forms at the site of administration of an increased anthraxin dose a well marked zone of hyperemia with a quite firm and deep infiltration of the skin. The thickness of the skin layer exceeds the norm 2.5-3.5 times.

Microscopic examination reveals the presence of an extensive process of inflammation in the dermis. The cell infiltrate, consisting mainly of neutrophile leucocytes, forms immediately under the epidermis some nests connected with one another (see Drawing 7 in original). A part of the leucocytes is in a state of disintegration. The epidermis over the focus of inflammation is infiltrated, edematous, its cells are in a state of discomplexization. In the deeper layers of the dermis there are considerable perivascular and more diffuse infiltrates.

Particular attention deserves a marked edema of the subcutaneous loose connective tissue (see Drawing 8 in original). The size of the areas of this edema markedly exceeds that of the area of inflammation in the dermis. Throughout the edematous zone the vessels are hyperemic, the endothelia of the small veins and of the capillaries are swollen. Round the vessels are situated tubular layers of neutrophile leucocytes and mainly of histiocytary elements. Side by side with these one observes also a more diffuse infiltration of the whole edematous tissue. At places one finds among the cells a considerable amount of eosinophile leucocytes.

The edema and the inflammatory infiltrates penetrate the underlying muscles and are found not rarely on the interior surface of the bundles bereft of fibers.

Thus the intracutaneous administration of anthraxin leads to a responsive inflammatory reaction in immune as well as in not immunized guinea pigs. In the latter this inflammatory reaction is slight, involving mainly the upper layers of the dermis, and is easily reversible. The administration of anthraxin to sensitized guinea pigs leads to a different process: the process of infiltration is more intensive and accompanied by a marked edema. Depending upon the amount of anthraxin administered the appearances of the patho-morphological changes are apt to vary: in the case of introduction of a moderate dose the process of inflammation may run its course with comparatively slight manifestations of exudation; in the case of an administration of increased anthraxin doses the edema and the infiltration of the subcutaneous layer are

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After 24 hours there forms at the site of administration of an increased anthraxin dose a well marked zone of hyperemia with a quite firm and deep infiltration of the skin. The thickness of the skin layer exceeds the norm 2.5-3.5 times.

Microscopic examination reveals the presence of an extensive process of inflammation in the dermis. The cell infiltrate, consisting mainly of neutrophile leucocytes, forms immediately under the epidermis some nests connected with one another (see Drawing 7 in original). A part of the leucocytes is in a state of disintegration. The epidermis over the focus of inflammation is infiltrated, edematous, its cells are in a state of discomplexization. In the deeper layers of the dermis there are considerable perivascular and more diffuse infiltrates.

Particular attention deserves a marked edema of the subcutaneous loose connective tissue (see Drawing 8 in original). The size of the areas of this edema markedly exceeds that of the area of inflammation in the dermis. Throughout the edematous zone the vessels are hyperemic, the endothelia of the small veins and of the capillaries are swollen. Round the vessels are situated tubular layers of neutrophile leucocytes and mainly of histiocytary elements. Side by side with these one observes also a more diffuse infiltration of the whole edematous tissue. At places one finds among the cells a considerable amount of eosinophile leucocytes.

The edema and the inflammatory infiltrates penetrate the underlying muscles and are found not rarely on the interior surface of the bundles bereft of fibers.

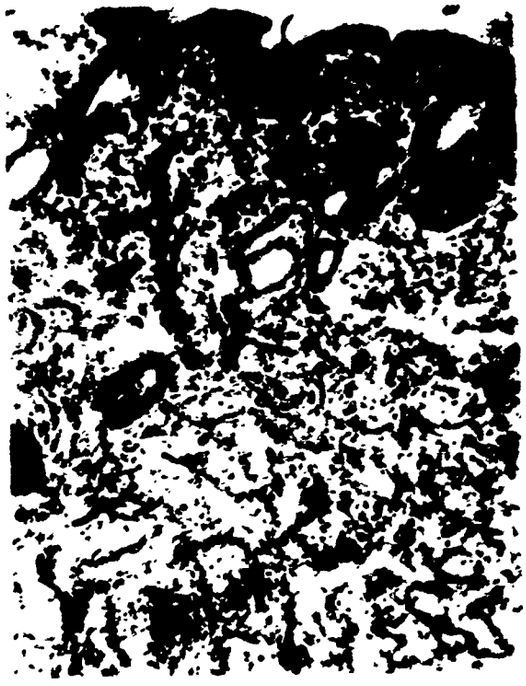
Thus the intracutaneous administration of anthraxin leads to a responsive inflammatory reaction in immune as well as in not immunized guinea pigs. In the latter this inflammatory reaction is slight, involving mainly the upper layers of the dermis, and is easily reversible. The administration of anthraxin to sensitized guinea pigs leads to a different process: the process of infiltration is more intensive and accompanied by a marked edema. Depending upon the amount of anthraxin administered the appearances of the patho-morphological changes are apt to vary: in the case of introduction of a moderate dose the process of inflammation may run its course with comparatively slight manifestations of exudation; in the case of an administration of increased anthraxin doses the edema and the infiltration of the subcutaneous layer are

marked; it comes to the formation of nests of purulent inflammation in the dermis and to necrosis of the skin at the site of administration to the preparate. It must be noted particularly that the edema becomes most marked in the loose subcutaneous tissue, extending over an area much larger than that of the focus of inflammation in the dermis.

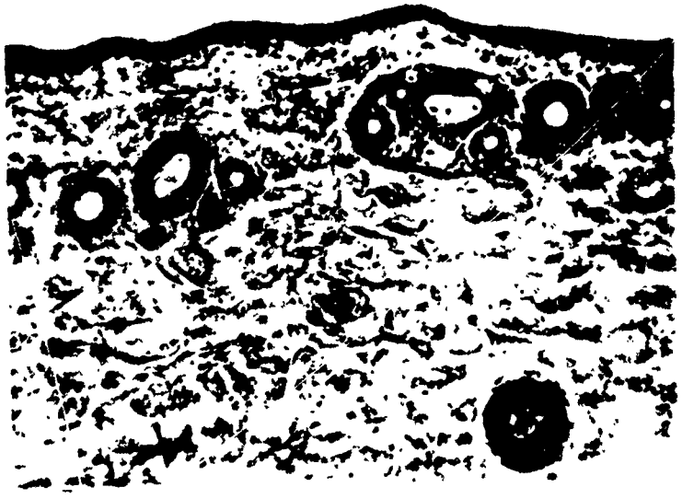
It is known that after intracutaneous administration of tuberculin, tularin or brucellin the ensuing process of inflammation indicates not only the immunological reactivity (sensitization) of the body but also some peculiarities of the specific morphological reaction characteristic for the organisms in question (formation of nodules, cell composition in the focus of inflammation, etc.).

In the case of anthrax the process of inflammation shows no specific morphological features; thus it is natural that the reaction to anthraxin as well is not characterized by any specific peculiarities. As in the case of other allergic reactions it is characterized by a proliferation of histiocytary and lymphoid elements and accompanied by inflammatory perivascular infiltrates.

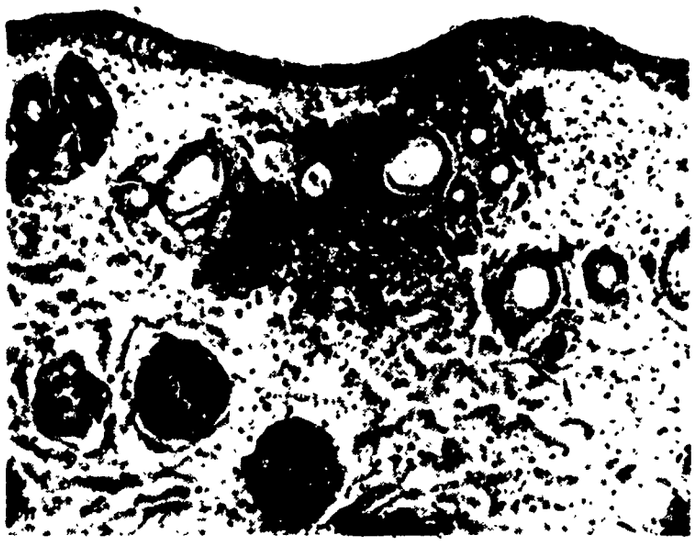
The only morphological peculiarity, well known to students of the inflammatory process in the case of anthrax, is the edema of the subcutaneous tissues. Its degree and extent are in accordance with the character of the reaction. The most extensive edema and inflammation, involving also the subcutaneous muscules, are met with after intracutaneous administration of large anthraxin doses.



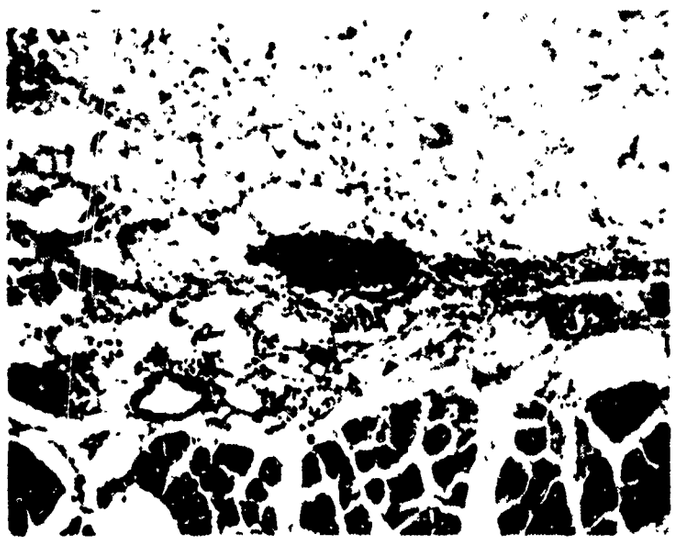
Drawing 1. Histological appearances 24 hours after anthraxin administration to a not immune guinea pig. Small perivascular infiltrates in the derma (x115).



Drawing 2. Appearances 24 hours after the administration of the control fluid: slight infiltration and edema of the upper layer of the derma (x135).

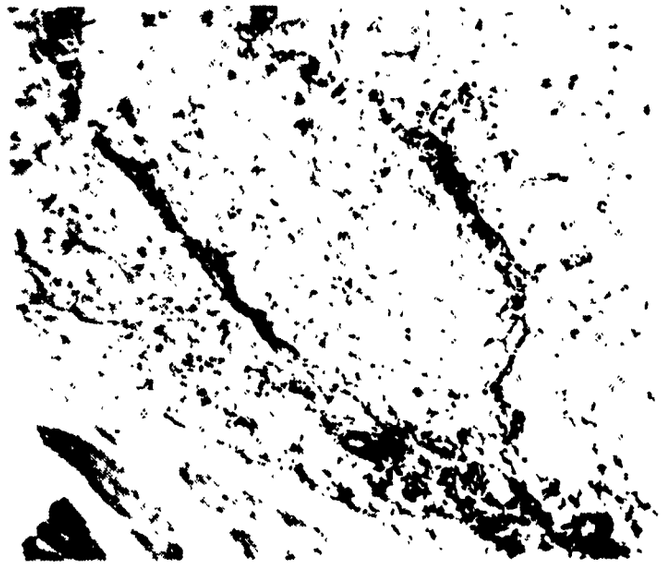


Drawing 3. Inflammatory infiltrate in the derma 24 hours after anthraxin administration to an immune guinea pig (x145).



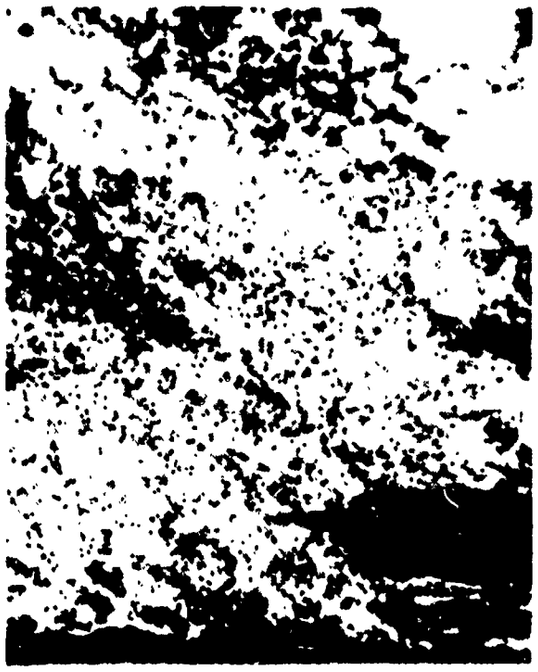
Drawing 4. Inflammatory infiltrate in the subcutaneous tissue 24 hours after anthraxin administration to an immune guinea pigs (x145).

Drawing 5. Perivascular infiltrates in the derma 24 (?) hours after anthraxin administration to an immune guinea pig (x245).



Drawing 6. Perivascular inflammatory infiltrates in the derma after 48 hours in the same animal (x120).

Drawing 7. Extensive focus of inflammation in the derma 24 hours after administration of an increased anthraxin dose to an immune guinea pig (x190).



Drawing 8. Marked edema and infiltration of the subcutaneous tissue after 24 hours in the same animal (x140).

(#94)

PRODUCTION OF STANDARD LIVE ANTHRAX VACCINE

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(Interdepartmental Commission of the Ministries of Health and Agriculture, USSR)

In order to prepare the proposed USSR standard specimen (reference preparation) of the live anthrax vaccine, from 1959 to 1962 studies were made of various lines of the vaccinal strain STI-1 and of various generations of its museum cultures. These investigations were made in comparison with other vaccinal anthrax strains.

Finally all requirements were met by a culture of the strain STI-1 (of N. N. Ginsburg), dried in 1943 from the third generation of the strain and kept since its isolation without subcultures.

The fundamental features of the thus received dry spore culture of the STI-1 strain which we propose to recommend as the USSR standard sample (reference preparation) for the control of the live medical anthrax vaccine were as follows: an examination of 10 ampulles (with an original volume of the suspensions of 0.5 ml) showed a residual moisture of 1%; the number of spores, counted in the chamber of Goriaev, amounted to 2 billion (± 10%) per ampulle; the number of live spores in microcultures amounted to 98%; the character of growth on agar was typical for the R-RO form of the colonies of B. anthracis; growth in broth took place in the form of floccules on the bottom, leaving the medium transparent; growth in serum-containing media led to the appearance of chains of uncapsulated organisms; no proteolysis took place on Schäfer's medium.

For an investigation of the immunogenicity of the anthrax vaccine on guinea pigs we used as a standard infective culture the variant 71/12 obtained from TSenkovskii Vaccine No. 2 in the form of a lyophilized spore culture. According to counts in the chamber of Goriaev the ampulles with an original volume of 0.5 ml contained 1.5 million ± 10% spores; the percentage of viable spores amounted to 98%. The character of growth on nutrient media corresponded to that of the original strain of the TSenkovskii Vaccine No. 2.

Further tests with the proposed standard sample of the anthrax vaccine STI-1 were made on white mice, guinea pigs, rabbits and sheep. The "residual virulence" of the sample was tested on white mice. Two series of tests were made on groups of 20 mice given each intracutaneously 10 million of spores in a volume of 0.1 ml.

After 2-3 days an extensive edema was noted in the majority of the mice and they succumbed after 6-10 days. Four mice of each group survived. Smears from the organs of the succumbed animals showed only uncapsulated bacilli.

The innocuousness of the samples was tested on rabbits. Twelve of these animals were used for the first test and 6 for the second. The animals were given each subcutaneously 250 million of spores of the vaccine suspended in 1 ml of normal saline. The rabbits were observed for 14 days. They all survived without showing a local reaction.

The immunogenicity of the proposed standard sample was tested on guinea pigs, rabbits and sheep. This work was done in a biofabrika by a commission jointly with the State Control Institute of Veterinary Preparations. Groups of guinea pigs were immunized subcutaneously with the STI-1 strain in doses of 1 million, 10 million, 25 million and 50 million suspended in 0.5 ml amounts of normal saline. The animals were challenged on the 15th day after immunization when tests with chemical anthraxin had been made.

Table 1

Results of Tests to Determine the Immunogenicity of the Proposed Standard Anthrax Vaccine on Guinea Pigs

Immunizing Doses of STI-1 Vaccine (Millions of Spores in 0.5 ml Saline)	Survival of Immunized Animals (2 Tests)	Tested with Chemical Anthraxin 14 Days after Vaccination			Challenge with Strain 71/12 in 0.5 ml Saline	Survived Challenge
		-	++	+++		
50	14/17	-	-	14	200 DCL	14/14
25	16/20	-	-	16	"	16/16
10	17/20	-	-	17	"	17/17
1	13/20	10	3	-	"	6/13*
Not immunized controls	-	7	-	-	"	0/7**

*) All guinea pigs weakly reacting to anthraxin survived.

**) The control animals succumbed within 72 hours.

As shown by this table, we obtained results confirming the good immunogenicity of the standard vaccine and the possibility of determining this in guinea pigs through challenge with cultures of the strain 71/12 of TSenkovskii's vaccinal strain No. 2 and allergic tests with chemical anthraxin.

Further evaluations of the immunogenicity of the proposed standard vaccine were made through tests on rabbits and sheep. 12 rabbits weighing 2.5 kg each were immunized subcutaneously with 250 million spores suspended in 1 ml quantities of normal saline, 7 sheep with 12.5 million doses of spores suspended in 0.5 ml quantities of saline.

The rabbits and sheep were challenged 14 days later with the highly virulent anthrax strain "CH-7" which had been titrated previously on 10 rabbits and 10 sheep.

Table 2

Results of Immunogenicity Tests with Proposed Standard Vaccine in Rabbits and Sheep

Kind of Animal	Number Tested	Immunizing Dose	Challenge Dose	Survived Challenge
Rabbits	12	250 million spores	10 DCL	12
Sheep	7	12.5 million spores	-"-	7

As the table shows, the proposed standard vaccine is highly immunogenic; it proved slightly reactogenic (one sheep showed a temperature of up to 40°C for one day).

Because of the data available in regard to the stability of the spore culture of the strain STI-1, kept in the dry state for 18 years, and the stability of the glycerol anthrax vaccine issued for practical use during a period of not less than two years we are able temporarily to assign a period of not less than three years as the time of validity of the reference preparation proposed by us (subject to prolongation after further research).

For an evaluation of the immunogenicity of the anthrax vaccines we proposed the following scheme: tests ought to be made on guinea pigs which must be healthy and weigh 400-450 g. The vaccine under test or the standard vaccine (reference preparation) are administered subcutaneously on the inner side of the leg in doses of 10 million and 25 million spores suspended in 0.5 ml amounts of normal saline without depot substances.

Taking into account the possibility of the death of a part of the animals after the vaccination, it is indispensable to use for each dose of the vaccine under test and of the standard vaccine not less than 12 animals. The death rate of the animals during the period of vaccinal infection ought not to exceed 4 out of the 12 animals of each group. If more animals die, they ought to be considered as unsuitable and a new group ought to be used for the tests. Fourteen days after the immunization cutaneous tests with anthraxin are made and on the 21st day after the immunization eight animals of each group are challenged with 200 LD_{50} (1 million spores) of the standard culture 71/12.

Thus 16 guinea pigs each will be available for an evaluation of the vaccine under test and of the standard vaccine. Not less than 4 guinea pigs ought to be used as controls.

As a rule, of the 16 guinea pigs not less than 80% (12-13 animals) ought to react positively to the anthraxin and all animals ought to survive. We consider it possible to permit the death of not more than 3 of the 16 guinea pigs immunized with the standard vaccine.

The vaccines under test ought to give no worse results than the standard samples. If so the vaccines under test may be considered as having passed the immunogenicity test.

Conclusions

1. A study of a dry culture of the vaccinal strain STI-1 permits the consideration of its use as the USSR standard preparation for testing the immunogenicity of live anthrax vaccines.

2. Likewise prepared and tested was a dry lyophilized spore culture of the variant 71/12 of TSenkovskii's variant No. 2, the use of which is proposed for challenge tests in guinea pigs in order to evaluate the immunogenicity of anthrax vaccines.

3. A scheme has been proposed for testing the immunogenicity of anthrax vaccines in guinea pigs with the aid of the standard vaccine, the standard culture for challenge and chemical anthraxin.

METHODS OF LABORATORY DIAGNOSIS AND IDENTIFICATION OF THE CAUSATIVE ORGANISMS

(#95)

DIAGNOSTIC VALUE OF SOME MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF THE ANTHRAX BACILLUS

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(Moldavian Institute of Epidemiology, Microbiology and Hygiene)

(Original pp. 124-138)

Improvements of the laboratory diagnosis of anthrax and the elaboration of accelerated methods for the identification of the organisms continue to be problems of actual importance.

Notwithstanding the simplicity of the cultivation of the anthrax bacilli, their characteristic growth on the generally used nutrient media and the availability of a large number of tests recommended for the identification of B. anthracis, its laboratory diagnosis is often fraught with difficulties.

On the one hand this is explained by the frequent natural occurrence of spore-forming saprophytic aerobes possessing morphological, tinctorial and cultural properties quite similar to those of the anthrax bacillus. Particularly difficult is a differentiation between B. anthracis and B. cereus. The similarity of these two microbial species is so close that some workers regard the anthrax bacillus as a pathogenic variant of B. cereus (31, 32, 45).

On the other hand a considerable phylogenic variability is peculiar for the anthrax bacillus. Studies on this problem have been the subject of many publications both Soviet and foreign authors. Variations of the morphological and cultural properties as well as of the virulence of the anthrax bacilli have been demonstrated (1, 2, 10, 14, 27, 28). It follows from these studies that it is not permissible to use for the differentiation of B. anthracis any single diagnostic sign and that is necessary to ascertain several signs of the organisms under test.

The generally used methods for the identification of the anthrax bacillus, described in the widely used medical and veterinary manuals (12,13,15,16,19,24,25,26, 30) fall fundamentally into the following three categories:

1) Microscopic examination of smears made from the native material;
3) Cultivation on nutrient media;
4) Animal experiments (biological tests).

Moreover in recent years many papers have been published dealing with the use of specific anthrax bacteriophages for diagnostic purposes. An evaluation of this method will be the subject of a separate publication.

For the convenience of record we propose to deal seriatim with the groups of signs and the methods of identification based on a consideration of these tests. Among the method based upon a consideration of the morphological pecularities of the anthrax bacilli one has to mention the bacterioscopic examination of smears (form), determinations of the motility of the organisms and accounts of their ability to form capsules.

Bacterioscopy. The presence in the smears prepared from the material under test of large uncapsulated and gram-positive bacilli is merely of orientative value since a large group of spore-bearing saprophytic aerobes shows the same morphological and tinctorial properties. Therefore this sign was found to be of no importance.

Motility. The most constant characteristic of B. anthracis is the absence of motility (16, 24, 31, 37, 42, 44). Still, a whole series of immotile variants of B. cereus has been described (31, 32).

Capsule formation. A sign invariably permitting to single out the anthrax bacillus among the large group of spore-forming aerobes is the presence of a capsule in the living organism. Only the anthrax bacillus forms a capsule in the living organism, while the saprophytic aerobes never do so (15,16,19,24,32).

The formation of a capsule by B. anthracis is possible also an artificial nutrient media but in this case the presence of a capsule has been observed also in variants of B. cereus (31). A number of methods has been proposed for growing capsulated bacilli in vitro. These comprise cultivation on serum agar and in an atmosphere of CO_2 (35, 42, 47). Virulent anthrax bacilli are able under these conditions to form capsules. Particularly good results can be obtained in this respect through cultivation in fluid serum (43) or in fluid serum to which Hanks' solution has been added (GKI medium (6), when the formation of capsules is observed 4-6 hours after inoculation of the media. The media of Thorne (48) and Buza (34) are suitable for the same purpose. Buza's medium is also suitable for a study of the hemolytic and hemopeptic properties of the organisms.

For a determination of the cultural and biochemical properties of the anthrax bacillus a number of nutrient media are used. Most widely used among them are meat-peptone agar (MPA) and meat-peptone broth (MPB). The outward appearances of the growth of the anthrax bacillus on MPA and MPB are as rule characteristic. IA. J. Koliakov (16) states: "The character of the growth of B. anthracis on MPB, MPA and MPZH is so typical that it may be taken into account in the differential diagnosis of this organism from other similar not pathogenic spore-forming aerobes." Besides MPA and MPB one uses for a differentiation of the anthrax bacilli from saprophytic aerobes 15-20% meat-peptone gelatin (MPZH) on which the anthrax bacillus grows in the form of a miniature pine tree turned upside down; the liquefaction of gelatin progresses slowly, ending on the 5th-65th day (12,13,15,16,19,24,32,33,43).

Besides the generally used determination of the proteolytic properties of the strains under test in gelatin tubes a method has been described of observing the proteolysis on gelatin agar (36) which according to the author is more objective and accurate than the observation of the proteolytic properties of the organisms on the the MPZH medium.

According to the statements in the literature the most constant biochemical characteristics of the anthrax bacillus are the absence of hemolytic properties (12,13,15,16,17,19,24,30,32, 43) which are ascertained with the aid of cultivation on blood agar or in blood broth and also the late coagulation of egg yolk, taking place according to M. V. Revo (23) and V. IA. Antonets(5) on the 3rd-4th day of incubation whereas the pseudo-anthrax bacilli and anthracoids coagulate egg-yolk after a growth for 6-8 hours. This property is inherent in an equal manner in virulent and avirulent strains.

Barber and Cury (1951) and afterwards Ivanovics and Foeldes (38) proposed a method of determining the production of an enzyme by the microbes which decomposed phosphate compounds. As a rule the phosphatase tests positive in the case of B. cereus and negative in that of B. anthracis.

A number of authors (5,6) points to the possibility of differentiating between anthrax strains and anthracoids with the aid of meat-peptone agar to which methylene blue has been added.

According to the statements of these authors virulent anthrax strains are endowed with reducing properties and grow (on this medium) in the form of yellow colonies whereas avirulent strains and anthracoids donot reduce methylene blue so that the color of the colonies remains unchanged.

The papers by Jensen and Kleemeyer (37), A.D. Melikhov (18) and Seidel (44) pointed to the possibility of a rapid diagnosis of anthrax with the aid of the "pearl string test" (Perlschnurtest).

The authors recommend this method as one of the constant differential-diagnostic tests permitting to detect the B. anthracis within 3-6 hours.

Some authors sucessfully used tests for penicillase for differential-diagnostic purposes (18,38). According to these investigators this test is fully specific.

For the differentiation of B. anthracis Seidel and Strassman (43) proposed the use of Petragnani's medium on which the saprophytic aerobes grew in the form of colonies with a white, yellow or brown color.

Many investigators showed that the spore-forming saprophytic aerobes, particularly B. subtilis, B. mesentericus, B. cereus, etc. are heat-stable, whereas B. anthracis loses the ability to multiply at a temperature above 45°C [Burdon (33)A.D. Melikhov (18) and others]. This property of the organisms was also used as a differential sign. According to the authors this test is extraordinarily easy to use and absolutely reliable; still it gives no idea in respect of the degree of virulence of the anthrax bacillus.

In the opinion of the majority of the competent investigators, the most accurate and irrefutable proof for the identification of an organism under test as anthrax bacillus is the demonstration of its pathogenicity for susceptible laboratory animals followed by identification of the causative organisms isolated or observed in the carcasses of the animals succumbed to the infection (7, 16, 24, 33, 40, 46).

In order to inhibit the phagocytic activiy of the experimental animals and to created better conditions for the formation of capsules in vivo Mesrobeanu and Slavesco (41) used for the inoculation of white rats anthrax cultures with an admixture of yolk emulsion which in their experience led to the death of animals usually resistant to the infection.

An analogous procedure was used by Klein, Mahlandt et al. (39) to increase the virulence of suspensions of anthrax bacilli administered to guinea pigs.

* * * * *

In the present paper we verified the above described methods of identification of the anthrax bacillus based upon the observation of various manifestations of the vital activity specific for this organism and attempted to work out the most simple, accurate, constantly practicable and at the same time reliable methods.

Materials and Methods

Strains under test. As checks on the tests and methods were used 55 strains of B. anthracis which according to their origin fell into the following categories: Of human origin -17; from cattle -4; from "small cattle" (i.e. sheep or goats) -2; from a horse - 1; from a pig -1; from the

soil of a burial ground of cattle -5; from imported raw material -12; of unknown origin -7; vaccinal strains -6.

The vaccinal strains were: STI-1 -2; SH-15 -1; "Indian" -1; TSenkovskii's 2nd vaccine -1; a strain of TSenkovskii's 2nd vaccine restored by V. R. Arkhipova (71/12) -1; (the last two strains were potentially capsulated).

46 of the strains were fully virulent while 3 were of attenuated virulence and not able to form capsules; two of them had been isolated from animals, one from man. Examined were also 29 strains B. cereus; 4 of B. anthracoides; 1 of B. pseudo-anthracis; 2 of B. mycoides; 3 of B. megatherium; 1 of B mesentericus vulgatus; 3 of B. subtilis; 1 of B. brevis - i.e. a total of 44 strains of spore-forming saprophytic aerobic bacilli.

The above mentioned strains had been obtained from local laboratories (Veterinary and Department of Specially Dangerous Infections of the Republic Sanitary-Epidemiological Station), the GKI* and TSNIDT+, the Rostov Anti-Plague Institute and the Kiev Institute of Epidemiology and Microbiology.

Tests studied. 1. The character of growth was studied in meat-peptone broth (pH 7.2-7.4) and on Hottinger's agar with an amino-nitrogen content of 105 mg %. The growth was inspected after an incubation at 37°C for 24 hours.

2. The motility was studied in hanging drop preparations made from 24 hour cultures of the organisms and also through inoculation of 0.3% MPA to which 0.005% of triphenyl tetrasolium chloride had been added (40). The motility was tested after an incubation at 37°C for 24-48 hours. In the presence of a motility of the strains one noted reddening of the whole medium or clusters of growth beyond the channel of inoculation.

3. The hemolytic and hemopeptic properties were studied by growing the organisms on plates of Hottinger's agar with 5% of fresh sheep blood and on agar kept in a 15% atmosphere of CO_2 (34). The cultures were incubated at 37°C, readings (determination of the zones of hemolysis and hemopepsis) were taken after 24, 48 and 72 hours.

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*) State Control Institute. Ed.

+) Central SR Disinfection Institute. Ed.

4. The proteolytic properties were studied through (a) cultivations of the organisms in tubes of 20% meat-peptone gelatin at 22° for 6 days. The character of the growth and the degree of the gelatin liquefaction were studied daily; (b) growth on the medium of Frazier consisting of NaCl 5 g; dipotassium phosphate-1.5 g; monopotassium phosphate - 0.5g; peptone-20g; agar 20g; gelatin - 4g; water - 600 ml. The medium was sterilized under a pressure of half an atmosphere and distributed into Petri dishes. Inoculations were made on the center of the plates so as to obtain gigantic colonies. After an incubation at 37°C for 72 hours a mixture consisting of 15 g sublimate and 2 ml HCl dissolved in 100 ml of distilled water was poured over the plates. After 2-3 minutes the excess fluid was poured off and measurements were taken of the diameter of the colonies and the width of the zone of clarification. The relation of these two figures was due to characterize the proteolytic properties of the organisms.

5. Lecithinase tests: The cultures under test were inoculated in the medium of Drozhzhevkina consisting of 1 part of egg yolk and 2 parts of normal saline. The ingredients were mixed under sterile precautions in a flask containing beads, the mixture was poured in 5 ml quantities into sterile tubes which were inoculated with loopfuls of a 24 hour old culture. Incubation at 37°C. Readings were taken after 12, 24, 48 and 72 hours.

6. Penicillinase tests were made with the aid of the inoculation of a 24 hour broth culture on plates of Hottinger's agar containing 50 and 10 units of penicillin per ml of the medium. The cultures were incubated at 37°C, readings were taken after 10, 24 and 48 hours (when the presence of growth was ascertained).

7. "Pearl string tests": The organisms were inoculated on blood agar plates which were incubated for 24 hours at 37°C. Then subcultures were made in tubes with meat-peptone broth which were incubated for 3 hours at 37°C. Loopfuls of the broth cultures were placed on MPA plates containing respectively 0.5 and 0.05 units of penicillin per ml while one plate without the antibiotic served as control. Plates with a surface area of 1 square centimeter and a thickness of 0.2 cm were incubated in a moist chamber at 37°C. After an incubation of 3 and 6 hours the plates were inspected under the microscope for the presence of a "pearl string", i.e. a chain consisting of anthrax bacilli changed into disks (L forms).

8. Reduction of: (a) malachite green on Petragnani's medium. The organisms were inoculated on slants (?) of the medium (IU. A. Kozlov, 1950). The cultures were incubated at 37°C, readings of the growths were taken after 24, 48 and 72 hours, when the size and the color of the colonies were noted; (b) methylene blue (medium of Borisov); this medium was prepared by adding to MPA melted and cooled to 45°C a watery solution of methylene blue in a proportion of 1:50,000; the medium was poured into Petri dishes. The character of the growth was studied after an incubation at 37°C for 24 hours.

9. Phosphatase test: The organisms were grown on plates with MPA to which was added 0.001 % of phenolphthalein phosphate. The plates were incubated for 24 hours at 37°C and then their surface was covered with some drops of concentrated ammonia water. The color of the colonies was noted.

10. Growth at 45°C: The organisms under test were inoculated on Hottinger's medium and the plates were incubated at 45°C for 72 hours. Observations of the growth were made after 24, 48 and 72 hours.

11. The formation of capsules in vitro was studied:
(a) with the aid of cultivation on 10% serum agar, incubated in an atmosphere of CO_2 (10-20%). The presence of growth and of capsulated organisms was observed after 6, 9 and 24 hours;
(b) with the aid of cultivation on plates with the medium of Thorne, Gomez and Housewright (48) incubated at 37°C. Readings of the growth and the presence of capsulated organisms were taken as in the case of serum agar;
(c) with the aid of cultivation on Buza's medium incubated in CO_2 atmosphere. The formula of Buza's medium has been given in paragraph 3 above. Readings were taken after 6, 12 and 24 hours.
(d) through cultivation on the GKI medium consisting of 60 ml of Hanks' solution and 40 ml of inactivated cattle serum, mixed under sterile precautions; after addition of soda (pH 7.2) the mixture was poured in 2 ml quantities into test tubes with rubber stoppers; incubation at 37°C, readings after 12 and 24 hours.

12. The pathogenicity and capsule formation in vivo was studied in intraperitoneally infected white mice (weighing 16-22 g). Infections were made with suspensions of B. anthracis (bacilli and spores) of different age - 3-5, 6-12 and 20-24 hours in the case of bacilli and 10-90 days in that of spores. The inocula were prepared in normal saline as well as in an egg-yolk emulsion according to Mesrobeanu and Slavesco (41). The density of the suspensions was determined at first according to the optical

standard of the GKI and then through direct counts of the chains (bacilli) and spores in the chamber of Goriaev. The density of the inocula of the bacilli grown for 6-24 hours varied from 5.5×10^6 to 5.5×10^8, that of 3-5 hour old growths from 2.9×10^4 to 2.1×10^6; that of the spores equalled 10^7. The inocula were injected in 1 ml quantities. In a number of tests the mice were infected with 24 hour old cultures of anthrax bacilli and of aerobic saprophytes, mixed either in equal parts or in a proportion of 1:3. The density of the inocula was 5.5×10^8.

The infected animals were kept under observation until they died; surviving animals were killed on the 10th day. The succumbed or killed animals were dissected; impression films were made from their internal organs and smears from the heart blood which after fixation were stained (1) according to Gram; (2)with an 1% solution of toluidin blue; (3) with fuchsine-methylene blue; (4) with Romanowski-Giemsa stain and (5) with old methylene blue. The stained preparations were inspected under the microscope in order to detect capsulated organisms.

The macroscopic pathological changes in the organs were also studied.

Results of the Investigations

Morphological Findings

1. Tests for motility, made with "hanging drop" preparations and in 0.3% agar proved not absolutely reliable though all 55 anthrax cultures were immotile, the same held true of 2 strains of B. cereus (strains P-29-2 and 555), while 1 strain B. pseudoanthracis (No. 24) and 1 strain of B. mesentericus vulgatus (85) were weakly motile (Table 1). The motility was recorded after 18-24 hours.

2. For tests for the formation of capsules in vitro we used (a) the medium of Thorne; (b) serum agar; (c) Buza's medium; (d) the GKI medium and (e) fluid cattle serum.

(a) On Thorne's medium were examined 47 anthrax strains, out of which 36 virulent strains grew in the "SM" form, showing capsulated bacilli in smears. At the same time 9 virulent strains as well as the not capsulated avirulent anthrax strains and the spore-bearing saprophytes grew in the "R" form and in the smears the organisms were not capsulated; this rendered a differentiation impossible (Table 1).

Morphological Characteristics

Table 1

Kind of Strains	Motility			Capsule Formation														
				Thorne's Medium			Buza's Medium			Serum Agar			GKI Medium			Fluid Serum		
	No. of Strains	+	-	No. of Strains	+	-	No. of Strains	+	-	No. of Strains	+	-	No. of Strains	+	-	No. of Strains	+	-
B. anthracis virulent	46	-	46	45	36	9	37	36	1	39	25	14	21	20	1	21	21	-
avirulent	9	-	9	2	1	1	9	2	7	7	1	6	7	2	5	6	-	6
B. cereus	29	27	2	29	-	29	29	-	29	-	-	-	10	-	10	10	-	10
B. subtilis	3	3	-	3	-	3	3	-	3	-	-	-	-	-	-	-	-	-
B. megatherium	3	3	-	3	-	3	3	-	3	3	-	3	-	-	-	-	-	-
B. mesentericus vulgatus	1	1+	-	1	-	1	1	-	1	1	-	1	-	-	-	-	-	-
B. brevis	1	1	-	1	-	1	1	-	1	-	-	-	-	-	-	-	-	-
B. mycoides	2	2	-	2	-	2	2	-	2	-	-	-	-	-	-	-	-	-
B. anthracoides	4	4	-	4	-	4	4	-	4	4	-	4	-	-	-	-	-	-
B. pseudo-anthracis	1	1+	-	1	-	1	1	-	1	1	-	1	-	-	-	-	-	-

(b) On serum agar in a CO_2 atmosphere out of 39 virulent anthrax strains 25 grew in the "SM" form and capsules were seen in the smears and 14 strains as well as the avirulent anthrax strains and the saprophytic aerobes grew in the "R" form and showed no capsules in the smears. Thus this method also gave unreliable results.

(c) On Buza's medium out of 37 virulent anthrax strains 36 grew in the "SM" form and showed capsules in smears; an exception was formed by one strain which, like the 7 uncapsulated avirulent strains, grew in the "R" form and showed in smears chains of uncapsulated bacilli. The spore-bearing saprophytic aerobes showed the same character of growth. Consequently with the aid of Buza's medium one could reliably differentiate the virulent anthrax strains from the avirulent and the saprophytic aerobes, since the one strain forming no capsules on this medium, when tested experimentally, proved to be weakly virulent (killing a mouse after 7 days). Readings of the tests on capsule formation with the aid of all above mentioned methods gave optimal results after 24 hours (Table 1).

(d) On the GKI medium capsule formation was noted in 20 out of 21 virulent anthrax strains as well as in 2 vaccinal strains (TSenkovskii-2 and 71/12); 1 virulent strain, 5 uncapsulate avirulent strains as well the 10 strains of saprophytes examined grew in the form of chains of uncapsulated bacilli.

(e) In fluid cattle serum capsule formation was observed in all 21 virulent anthrax strains, while 6 avirulent strains and 10 strains of spore-bearing saprophytes showed no capsules. With the aid of these two methods one could read the results within 10-12 hours.

Cultural Characteristics

(a) The character of growth on MPA in the ordinary atmosphere was typical in the overwhelming majority of the anthrax strains, i. e. growth took place in the form of fluffy silver-white colonies (R form) with a wavy outline; an exception was formed by one strain (No. 12) which formed round colonies with an even outline. The strains of B. cereus resembled the anthrax bacillus in the character of their growth except that in their case many curls jutted out from their outline. The same character of growth was shown on the MPA medium by B. anthracoides and B. pseudoanthracis. All other soil saprophytes markedly differed in respect to their growth on MPA from the above described type (Table 2).

(b) In MPB the majority of the anthrax strains grew in a form resembling cotton flakes at the bottom of the tubes while the medium remained transparent; only 2 strains (98, 15) showed a slight ring adherent to the wall of the tubes which could be washed off easily.

All spore-forming saprophytes either rendered the broth turbid or produced flakes or differed in respect to the character of the sediment (B. brevis); only 2 strains of B.cereus (P-29-2 and 555) produced in the broth the growth characteristic for B. anthracis (Table 2).

(c) The optimal temperature for the growth of B. anthracis was 37° and growth was absent at a temperature of 45°-45.5°C; the saprophytic spore-bearing saprophytes showed (at these temperatures) already after 10-14 hours a luxuriant growth. Still, as in the preceding cultural tests, a number of B. cereus strains (Nos. 79, 398, 614, 104 P, 379, 17., 372, 325 and 363) and 1 strain of B. megatherium (77) behaved like B. anthracis, i.e. they did not grow at these temperatures (Table 2). In all cultural tests readings could be taken not earlier than after 18-24 hours.

Table 2.

Cultural Properties

Kind of Strains	No. of Strains	MPA Typical*	MPA Atypical*	MPB Typical*	MPB Atypical*	Growth at 45°C +	Growth at 45°C -
B. anthracis							
virulent	46	46	-	45	1	-	46
avirulent	9	8	1	8	1	-	9
B. cereus	29	-	29	2	27	20	9
B. subtilis	3	-	3	-	3	3	-
B. megatherium	3	-	3	-	3	2	1
B. mesentericus vulgatus	1	-	1	-	1	1	-
B. brevis	1	-	1	-	1	1	-
B. mycoides	2	-	2	-	2	2	-
B. anthracoides	4	-	4	-	4	4	-
B. pseudoanthracis	1	-	1	-	1	1	-

*) For B. anthracis

Table 3

Table 3

Biochemical Properties

Kind of Strains	Growth on MPZH				Hemolysys			Coagulation of Egg-Yolk			Formation of Phosphatase			Relation to Penicillin (Growth)			"Pearl-string" Test			Reduction of Methylene Blue			Reduction of Malachite Green**		
	No. of Strains	Typical*	Atypical*	Liquefaction	No. of Strains	+	-	No. of Strains	+	-	No. of Strains	+	-	No. of Strains	+	-	No. of Strains	+	-	No. of Strains	+	-	No. of Strains	+	-
Bacillus anthracis Virulent	42	39	3	S	45	1	44	37	-	37	45	1	41 3±	46	-	41 5±	46	46	-	33	33	-	45	45	-
Avirulent	3	2	1		9	-	9	3	-	3	7	-	5 1+ 1±	9	-	9	2	2	-	7	7±	-	9	9	-
cereus	29	-	29	25R 4S	29	25	4	29	24	5	29	28	1	29	24 1+	4	29	-	29	29	8	21	29	16	13
subtilis	3	-	3	S	3	2	1	3	-	3	3	3	-	3	2	1	3	-	3	3	-	3	3	-	3
megatherium	3	-	3	S	3	1	2	3	-	3	3	3	-	3	2	1	3	-	3	3	-	3	3	-	3
mesentericus vulgatus	1	-	1	O	1	-	1	1	1	-	1	1	-	1	1	-	1	-	1	1	-	1	1	-	1
brevis	1	-	1	O	1	-	1	1	1	-	1	1	-	1	1	-	1	-	1	1	-	1	1	-	1
anthracoides	4	-	4	R	4	4	-	4	4	-	4	4	-	4	4	-	4	-	4	-	-	-	4	-	4
mycoides	2	-	2		2	1±	1	2	-	2	2	2	-	2	2	-	2	-	2	-	-	-	2	-	2
pseudo-anthracis	1	-	1	R	1	1	-	1	1	-	1	1	-	1	1	-	1	-	1	-	-	-	1	-	1

* For B. anthracis
** Petragnani's Med.

S = Slow; R = Rapid; O = non-liquefying

Biochemical Properties

(a) A study of the tests for gelatinase with the aid of cultivation on MPZH at an incubation temperature of 22°C showed that not all anthrax strains gave a typical growth.....(an exception being formed by the virulent strains No. No. 100, 103, 104). Moreover, not all B. cereus strains rapidly liquefied gelatin, as is characteristic for the saprophytic aerobes (Table 3). Readings of the tests were made up to the 6th day of incubation.

Exploring the gelatinase test on Frazier's medium it was not possible to detect any regularity of the relation between the diameter of the colonies and the width of the zone of clarification (depending upon the hydrolysis of the gelatin by the organisms) which would permit a differentiation between the anthrax bacilli and the saprophytes. Moreover, the results of tests on this medium were variable (readings taken after 72 hours).

(b) A determination of the hemolytic properties on % blood agar showed that 53 anthrax strains failed to produce hemolysis after an incubation of 24 hours; and exception was formed by the virulent strain No. 88, producing a slight zone of hemolysis. Later readings led to the detection of zones of hemolysis in 9 anthrax strains. It detracted from the vaule of this test that out of 29 B. cereus 25 produced hemolysis but that 3 strains (Nos. 625, 313-T, 325) failed to do so while Strain No. 655 produced slight hemolysis after 48 hours.

An absence of hemolytic properties characterized a whole series of the other species of spore-bearing saprophytes (Table 3).

(c) The ability of the organisms under test to coagulate egg-yolk was tested in 40 anthrax strains (37 virulent, 2 vaccinal and one avirulent); all proved negative in this respect even after an observation period of 6 days, while the overwhelming majority of the saprophytes coagulated egg-yolk within 10-14 hours. An exception was formed by 13 strains of saprophytic aerobes which did not coagulate egg-yolk throughout the observation period of 6 days (Table 3).

(d) Phosphatase tests, made with 52 anthrax strains gave negative results with the exception of 3 virulent strains (Nos. 29, 103, 104) and 5 avirulent strains. However, the reactions in these cases had to be evaluated as "+" or "±" and only in the case of the virulent strain No. 86 was a +++ reaction obtained. On the other hand the colonies of the saprophytes, intensively producing phosphatase, showed an intensive pink color. With the exception of 1 B. cereus strain their reactions had to be classified as "++" or "+++".

(e) Sensitivity to penicillinase: Penicillin in doses of 10 and of 50 units per ml of the media exerted a bacteriostatic action on all 49 anthrax strains and only in 5 instances one could observe the growth of single colonies (1-2) on agar containing 10 units of penicillin per ml of the media. In the majority of the strains of B. cereus and of the other spore-bearing saprophytic aerobes one observed an abundant growth on the media containing 10 or 50 units of penicillin per ml after 10-14 hours; still, in this group of organisms also 6 strains behaved like B. anthracis, i. e. they did not grow on penicillin containing media, while one strain (No. 555) grew only on the media containing 10 units of penicillin per ml. (Table 3).

(f) An evaluation of the "pearl string" test showed that all 48 anthrax strains examined in this respect (virulent as well as avirulent and vaccinal strains) grew already after 3 hours on agar containing small doses of penicillin in disk form, whereas the spore-bearing saprophytic aerobes grew in the usual form (Table 3). The results were read after 6 hours.

(g) On Petragnani's medium the anthrax strains grew in the form of round bulging colonies of "ivory" color, but at the same time 16 out of the 29 B. cereus strains under test grew in the form of colonies not distinguishable or only with difficulty distinguishable from those of B. anthracis. Marked difference in their coloration were shown by the colonies of the other spore-bearing saprophytes which had a white, apple-green, yellow or brown color (Table 3). Observations on the character of the growth on this medium were made in the course of 6 days.

(h) On methylated agar the 33 virulent anthrax strains under test caused a reduction of methylene blue and grew in the form of yellow colonies whereas the avirulent and vaccinal strains and 29 strains of spore-bearing saprophytes, which did not reduce methylene blue, grew in the form of colonies of a blue color. Still, 8 strains of B. cereus also reduced methylene blue and their colonies did not differ from anthrax colonies (Table 3). Final readings in the case of this test were taken after 48 hours.

Results of Animal Experiments

The administration of watery suspensions from 20-24 hours old cultures of 27 anthrax strains of various virulence to 62 mice led in all cases to the death of the animals after intervals ranging from 17 hours (2 cases) to 72 hours (2 cases), as a rule after 20-24 hours (40 cases). The morbid findings were not in full accord with the classical description: there was no hyperplasia of the spleen and liver, specially not in the case of an early death, the blood was not coagulated and the presence of an anthrax sepsis could be confirmed in all cases through the observation of incapsulated organisms in the smears and impression films from the organs and the heart blood.

In 5 experiments with avirulent anthrax strains the animals (10 mice) survived and were killed. Bacteriological findings were negative.

The use of 6-12 hour old growths obtained from 4 virulent anthrax strains on 12 mice led in all cases to bacteriologically confirmed deaths from anthrax sepsis after intervals from 20 to 36 hours. The morbid findings were identical with those in the first series of experiments.

3-5 hour old cultures of 28 anthrax strains of various virulence led to the death of 60 white mice after intervals from 17 (1 case) to 72 (1 case) hours after the infection and as a rule after 20-24 hours (36 mice). The morbid findings did not differ from those described above.

In experiments with anthrax spores (10 strains) death of the animals (20 mice) from specific sepsis took place after intervals from 15 hours (1 case) to 48 hours (4 cases), most often after 36-48 hours (8 mice). Macroscopically the same atypical morbid findings were noted.

The administration of 24 hour old suspensions from 15 anthrax strains of various virulence in yolk emulsion (adjuvant) led to the death of the 44 mice under test after intervals from 20 to 48 hours, mainly after 24 hours (36 out of 44 mice). The macroscopic findings were not more marked than in the series of experiments in which suspensions of organisms of an analogous concentration and of the same age had been made in normal saline.

Animals infected with suspensions of spore-bearing saprophytes and uncapsulated anthrax strains either survived and were killed on the 10th day (when no organisms were found in impression films) or succumbed after 24-48 hours, when uncapsulated gram-positive bacilli were found in impression films from the internal organs.

In eleven experiments with mixed cultures of anthrax bacilli and spore-bearing saprophytic aerobes deaths from anthrax were observed with the exception of one experiment in which use had been made of a mixture consisting of a culture of an attenuated anthrax strain and a B.cereus culture. In this case the mice survived and were killed on the 10th day. Impression films from the internal organs did not show the presence of a microflora.

Evaluation of the Results

As a result of the experiences gathered it becomes clear that it is not possible to select any single one of the tests made for a differentiation of the virulent B. anthrax strains from attenuated strains and the saprophytes, since no single test fulfils the fundamental requirements, namely absolute reliability in combination with expedience. Thus the use of MPA and MPB as differential-diagnostic media can not be always reliable, the behavior of virulent and avirulent anthrax strains being quite identical. Analogous statements have been made also in the articles of a number of authors (8,16,25,26,29,32,43). The stability of one of the fundamental characteristics of B. anthracis, its immotility, has been confirmed by our experiences with the exception of 2 B. cereus strains in which motility was absent, while one strain of B. pseudoanthracis and one of B. mesentericus vulgatus gave doubtful results.

These findings are in accord with the statements of Burdon (33), Seidel and Strassman (43) and Jensen and Kleemeyer (37)who reported that while they did not observe motile anthrax strains, up to 30% of the saprophytic strains were also immotile.

Hemolysis tests gave constant results in the 53 anthrax strains examined in this respect and only one strain proved lytic for sheep erythrocytes (readings after 24 hours). At the same time, however, 10 strains of saprophytic aerobes also proved non-hemolytic, thus rendering this test doubtful. Analogous statements have been furnished by Burdon (33), Seidel and Strassman (43). Identically inaccurate results were obtained also in tests with Petragnani's medium and methylene agar.

A more constant characteristic of the anthrax bacilli was an absence of the ability to coagulate chicken egg-yolk but it detracts from the value of this test that 13 strains of saprophytic aerobes also failed to coagulate egg-yolk.

Growth on meat-peptone gelatin as well as tests for gelatinase on Frazier's medium gave by no means constant results; the length of the observation period required for the former test renders it little suitable for practical diagnostic purpose.

Phosphatase tests, cultivation at high temperatures (45.5°C) and penicillinase tests proved more reliable but can be used only with pure cultures; readings can be taken not before 24 hours; virulent and avirulent strains behave identically. A favorable evaluation of these tests has been made by Brown and Moody (32), Burdon (33), Seidel (44) and others.

A characteristic permitting in any case a differentiation between virulent (capsule-forming) anthrax strains and the voluminous group of spore-forming aerobes is the formation of capsules by the organisms in vivo or in vitro (10, 11, 13, 14, 18, 23, 40, 42). According to our experiences most suitable for the tests in vitro was cultivation on the medium of Buza (34) and on the GKI medium. Results of capsule formation on these media were more stable than those obtained with fluid or solid serum-containing media or with Thorne's medium.

Most valuable proved the tests for pathogenicity and capsule formation in vivo - the biological test. Positive results were obtained in all cases with one exception where use was made of a mixture of cultures of an attenuated anthrax strain and a B. cereus strain.

Shortcomings of this method in its "classical" form are the long time required for its performance, the indispensability of using pure cultures so as to avoid the appearance of a microbial antagonismus in vivo and the danger of an intercurrent gaseous infection. This series of factors materially detracts from the value of this in other respects fully sensitive and reliable method for the identification of virulent anthrax bacilli.

These difficulties, in the first line the length of the course of the septic process, could not be overcome by the use of egg-yolk emulsions which in general enhance the virulence of the suspensions of B. athracis.

Conclusions

1. Most reliable among the numerous methods for the identification of the anthrax bacilli proved the tests for the production of phosphatase and penicillinase (50 units per ml), hemolysis tests, pearlstring formation and cultivation at a high temperature (45° C). Still, all these methods and tests did not allow a distinction between virulent and avirulent anthrax strains.

2. An identification of virulent anthrax bacilli, based upon the phenomenon of incapsulation of the organisms and their pathogenicity for animals can be made with great accuracy with the aid of biological tests in susceptible laboratory animals (white mice) as well as through cultivation on Buza's medium or on the GKI medium.

3. In its generally used "classical" form the biological test for anthrax has a number of shortcomings, mainly the long duration of the observation period. This renders it indispensable to improve the method (through abbreviation and simplification).

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(#96)

ACCELERATED IDENTIFICATION OF PATHOGENIC ANTHRAX BACILLI WITH THE AID OF THE BIOLOGICAL TEST FOR INCAPSULATION

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In the preceding communication it has been shown that most convincing method for the identification of the anthrax bacillus is the biological test for pathogenicity, performed through inoculation of the native material or of suspicious cultures into laboratory animals and observation of their death from anthrax sepsis. It must be emphasized in this connection that the specificity of the death of the infected animals is determined with the aid of a microscopical examination of smears from the blood and of impression films from the organs through observation of gram-positive incapsulated anthrax bacilli. On this basis the presently accepted rules permit a final answer (1, 3).

The bacterioscopy is followed by a macroscopic examination of the morbid appearances and by cultivation from all parenchymatous organs in order to obtain pure cultures of B. anthracis. The results of these investigations merely confirm the initial bacterioscopic findings of incapsulated organisms.

Nevertheless the biological tests have a number of shortcomings, most important among which is the length of time required for their performance (see p. 88).

It has to be noted in this connection that, as shown by a study of the phenomenon of the incapsulation of the anthrax bacillus in vivo (7) in the plasma or serum of some animals, this process takes place during 30 minutes or less. This induced us to investigate through in vivo experiments the time of the appearance of capsule formation by virulent anthrax bacilli and also to study under the same conditions the potenially uncapsulated anthrax bacilli, including the vaccinal strains, as well as a large number of saprophytic species of aerobic bacilli.

In order to enhance the virulence of some microbes it has been recommended to use adjuvants like mucin or egg-yolk (12, 13). These adjuvants have been used also to increase the virulence of the anthrax bacillus (9, 11).

Usually infective doses of the materials under test (microbial cultures) are administered to the laboratory animals subcutaneously. Still, it has been shown (15) that intraperitoneal inoculation results in a more rapid absorption of the material; virulent organisms rapidly multiply in the peritoneal cavity (10). Through the recent work of Fernelius et al. (8) who used the method of assessing the average time of death of the animals as a means of determining the virulence of B. anthracis it has been shown that the intraperitoneal administration of virulent anthrax spores leads to a more rapid death of the animals than subcutaneous infection with the same doses.

Moreover, the intraperitoneal infection has a number of technical advantages in respect to accuracy and simplicity.

Material and Methods

I. Animals: Ordinary guinea pigs (300-600 g) and white mice (16-22g).

II. Strains tested: The same as described in the preceding report (see p. 88).

III. Volume and character of the inoculum: 1) Volume of the inoculum: For guinea pigs corresponding concentrations of the organisms were administered in 5 ml amounts of 0.85% normal saline or of an adjuvant; in the case of white mice the bacterial suspensions were administered in a volume of 1 ml; 2) Character of the inoculum: (a) Suspensions of 24 hour old organisms washed off from the surface of MPA with normal saline, the concentration being determined according to the GKI optical standard; (b) Suspensions of spores in normal saline, their concentration being determined through counts in the chamber of Goriaev; (c) Suspensions of spores in normal saline (their amount being ascertained through counts in the chamber of Goriaev) which before infection

of the animals were grown for 3-6 hours in MPB; (d) 5 hour old broth cultures with an unknown inoculating dose but in which the numbers of vegetative cells had been counted in the chamber of Goriaev; (e) Mixed cultures were prepared through mechanical mixture of two suspensions made from 24 hour old agar cultures of various strains in normal saline; the cell concentration was determined according to the GKI optical standard.

IV. The adjuvant was prepared according to the method of Pang (13): the viscosity of the egg emulsion equalled 14.5 units.

V. Performance of the accelerated biological tests:

(1) Method of puncturing the peritoneal cavity: Identified microbial suspensions were administered intraperitoneally to guinea pigs or white mice. After predetermined intervals of time (1, 2, 3, 4, 5 and 6 hours) the peritoneal exudate was aspirated with the aid of a sterile syringe and smears were made on slides. The smears were fixed with the mixture of Nikiforov, stained according to Gram's method or with diluted fuchsine and methylene blue and subjected to microscopic examination.

(2) Method of killing the animals developing the infection: An indentified microbial suspensions was administered to white mice, as a rule to 8, 10 or 16 animals, which in pairs were killed respectively after 30 minutes, 1 hour, 2 and 3 hours. For the purposes of control 2 mice were kept under observation until their death or, if they did not succumb, were killed on the 10th day.

In a number of experiments the infective doses were administered also subcutaneously and the animals were killed seriatim as stated above.

From the successively killed animals smears were made from the peritoneal exudate and the heart blood as well as impression films from the spleen, liver and kidneys. These preparations were fixed and stained as described above and examined microscopically.

The animals succumbing to the infection or killed after 10 days were dissected, the morbid appearance were studied and smears and impression films were made in the manner described above for microscopical examination.

In the course of the microscopic examination attention was paid to the presence and character of the microflora and - if bacillary forms were observed - to the presence of capsules and the position of the organisms in the tissues.

Results of the Investigations

Experiences with puncture of the peritoneal cavity. Preliminary tests made with intraperitoneal administration of microbial suspensions to 12 mice followed by punctures of their peritoneal cavity so as to obtain and examine the exudate showed the impracticability of this method. The mice badly tolerated the repeated punctures of their peritoneal cavity; moreover each (successive) puncture increased the danger of damaging the tissues of this cavity and the possibility of an introduction of the material into the intestinal tract; on the other hand the amount of the exudate became insufficiently restored during the intervals between the punctures.

Of the total number of mice under test, 5 developed an unspecific peritonitis leading to a disturbance of the usual features of anthrax infection. For this reason the further use of this method in mice was discontinued.

For a further use of the method of periodical puncture of the peritoneal cavity tests were made in larger animals - guinea pigs. These tests were made in a group of animals to which suspensions of 24 hour cultures (in a dose of 5.5×10^8) with an adjuvant were administered intraperitoneally.

The results of the tests in guinea pigs are recorded in Table 1. Guinea pig No. 1 received a weakly virulent anthrax culture, animals 2, 3, 4 and 5 virulent cultures.

The results of these tests show that it is possible with great constancy to observe with the aid of periodical punctures of the peritoneal cavity in the exudate incapsulated virulent anthrax bacilli 3-4 hours after administration of the suspensions.

The results of an examination of the smears made from the exudate were in full accord with the findings made after the animals in question had succumbed to anthrax.

Experiences in Animals Killed during the Development of the Infection

In the first series of these tests use was made of virulent anthrax cultures in the vegetative form, suspensions of which were administered to white mice by the intraperitoneal route. The results of these investigations are shown in Table 2.

The data of this table permit to postulate an interrelation between the concentration of the inoculum (infective dose), its age and the rapidity with which indicatory data are obtained and also permit a comparison of the efficacy of this test with that of the classical biological tests.

The results of the 3rd and 6th series of the tests allow characterization of the aptitude of the proposed method. Thus already in the course of the first hour after the intraperitoneal administration of young (3-5 hour old) cultures in average doses of some hundred thousands (0.29-2100 thousand organisms) a positive result was obtained in 9.7 out of 10 cases and after 2 hours in 10 out of 10 cases. As shown by Table 2, the indicators of the rapidity of incapsulation of young cultures are perceptibly higher than is the case in in tests with "adult" 24 hour cultures, introduced in considerably higher amounts (ten times higher).

In these tests in the course of the first hour out of possible 10 only 6.8 positive results were obtained while at the end of the second hour after infection positive reactions were noted in 100%.

Table 1

Results of Examination of Punctates from Non-immune Guinea Pigs

Species of Cultures Used	Number of Animals Used	Results of Examination After 1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	Times of Death	Times of Killing	Results of Examination of Succumbed or Killed Animals
B. anthracis No. 18	1	-	-	-	++	-	-	96 hrs.	-	-
No. 30	2	++	++	++	++	++	++	24 hrs.	-	++
No. 29	3	++	++	++	++	++	++	48 hrs.	-	++
No. 28	4	++	++	++	++	++	++	48 hrs.	-	++
No. 4	5	-	++	++	++	++	++	36 hrs.	-	++
B. anthracoides No. 22	6	-	-	-	-	-	-	-	10 days	-
No. 22	7	-	-	-	-	-	-	-	10 days	-
No. 23	8	+	+	+	+	+	+	-	10 days	-
No. 23	9	+	+	+	-	+	+	-	10 days	-

Explanation of signs: ++ = Incapsulated gram-positive bacilli;
+ = Not capsulated gram-positive bacilli;
- = No bacilli found.

Table 2

Incapsulation of B. anthracis at Different Intervals after Intraperitoneal Infection of White Mice

Series of Tests	Number of Strains Examined	Age (Hours)	Concentration	Time of Incapsulation (Minutes) 30	60	30-60	120	30-120	180	30-180	Time of Death of the Animals (Hours) M ± m	sigma
1	27	20-24	5.5×10^8	9/24*	21/31	21/31	28/31	31/31	14/14	31/31	28.2 ± 0.88	± 6.9
2	4	6-12	5.5×10^6-10^8	Not tested	6/6	6/6	6/6	6/6	6/6	6/6	20.5 ± 1.43	± 5.0
3	28	3-5	2.9×10^2 - 2.1×10^6	13/29	31/32	31/32	32/32	32/32	31/31	32/32	24.2 ± 1.26	± 9.62
4	2	3	0.5×10^2-10^3	Not tested	0/9	0/9	0/9	0/9	0/9	0/9	82.3 ± 22.76	± 10.1
5	2	3**	0.5×10^2-10^3	-"-	0/10	0/10	0/10	0/10	0/10	0/10	48 ± 0	± 0
6	2	6	0.5×10^2-10^3	-"-	6/6	6/6	6/6	6/6	6/6	6/6	30.0 ± 6.05	± 12.13
7	2	6**	0.5×10^2-10^3	-"-	1/10	1/10	1/10	2/10	Not tested	-	52.8 ± 3.18	± 10.1
8	10	Spores 10- 90 days	10^7	0/10	2/10	2/10	8/10	8/10	10/10	10/10	30.6 ± 1.56	± 7.0

*) Numerator - Number of positive findings
Denominator - Number of pairs of mice examined

**) Infected subcutaneously

Thus the best results were obtained with suspensions of young organisms. It must be emphasized in this connection that the virulence of the strains used in the 1st and the 3rd series of tests was almost identical as confirmed by the slight differences in the time of death of the control animals. Consequently the more rapid capsule formation by young cultures must be ascribed to their biological activity which undoubtedly is more marked than that of 24 hour old cultures in which one finds often involution forms and resting forms (spores).

The efficacy of the biological test for incapsulation (in respect to time) has been calculated in relation to the time of death of the control animals. In the tests made (Table 2) absolutely reliable results have been obtained at the end of 2 hours after infection. This time interval is 7-17 times shorter than that required to obtain results with the aid of the classical biological test.

The administration of small doses of the infective suspensions of B. anthracis (50, 100, 200, 500 and 1,000 spores per ml) to mice after subcultivation of the organisms in MPB for 3 hours gave negative results both in the case of intraperitoneal and subcutaneous infection of the animals. Identical results were obtained in 8 tests (Table 2, series 4 and 5). Tests made with subcutaneously introduced suspensions of anthrax spores in the same concentrations after preliminary subcultivation in MPB for 6 hours gave a negative result (Table 2, series 7).

Intraperitoneal infection of the animals with the same initial doses but after subcultivation in MPB for 6 hours led to the detection of incapsulated organisms in the peritoneal cavity and in the majority of the internal organs already 60 minutes (and also 120 and 180 minutes) after infection of the mice with the suspensions, followed by killing of the animals at the times stated. These findings were confirmed in 2 tests (Table 2, series 6).

The marked biological activity of young bacilli induced us to perform a series of tests in which the infecting suspensions consisted not of vegetative forms of the organisms but of spores (Table 2, series 8).

As the results indicate, one hour after inoculation in only 2 (out of 10) instances the spores developed into incapsulated vegetative forms; after two hours this was the case in 8 out of 10 cases and at the close of 3 hours in all instances. Under these circumstances the rapidity with which results were obtained was still greater, inasmuch as the minimum of 3-5 hours necessary for the development of the spores was excluded - as was done for instance in the 6th series of tests (Table 2).

In Table 3 are assembled the data on the frequency with which incapsulated anthrax bacilli were found in the different organs and tissues of intraperitoneally infected mice in relation to the time of killing of the animals.

The table shows that the highest frequency with which regardless of the nature of the inoculum (its age or the form of the organisms - vegetative or spores) incapsulated anthrax bacilli were found, was noted at the site of inoculation, i.e. in the peritoneal exudate. In the second place stands the spleen, followed in decreasing order by the liver and kidneys; findings were most rare in smears from the heart blood.

Attention has to be paid to the marked biological activity of young cultures (3 series of tests) which in comparison with 24 hour old cultures (1 series of tests) are endowed with greater invasiveness or aggressiveness. These properties enable the young cultures to bring about in the course of 2 hours after infection an invasion of all investigated organs and tissues; in one third of the cases the organisms were isolated also from the heart blood, thus indicating a state of sepsis in the infected animals.

The rapidity and regularity of the invasion of the organs and tissue by the anthrax bacilli, specially by young cultures, after intraperitoneal infection convincingly show how much one must consider the method of biological tests in mice followed by observation until their death as antiquated and irrational, since at the same time all data necessary for a diagnosis can be obtained some tens of hours earlier.

A series of 9 tests dealt with the administration to white mice of inocula prepared from 5-24 hour old cultures of soil saprophytes (B. cereus, B. pseudoanthracis, B. anthracoides). The concentration of the suspensions administered to the animals varied from 14 million to 1 billion organisms per dose. Following the usual method of sacrificing the animals under test it was not possible to find incapsulated organisms in the organs and tissues. As a rule the controls survived up to the end of the observation period (10 days) and were then killed. Only in one case in which a massive dose of a 5 hour old culture of B. anthracoides No.76 had been administered, the animals died after 96 hours. But in this case also non-capsulated gram-positive bacilli were found at the autopsy.

Table 3

Occurrence of Incapsulated Anthrax Bacilli in the Various Organs of Mice, Infected Intraperitoneally

	Within 30 Mins. Series of Tests**			Within 60 Mins. Series of Tests			Within 120 Mins. Series of Tests			Within 180 Mins. Series of Tests		
	1	3	8	1	3	8	1	3	8	1	3	8
Peritoneal cavity	7/24*	5/29	0/10	16/31	27/32	1/10	29/31	31/32	8/10	14/14	29/31	10/10
Spleen	2/24	8/29	0/10	5/31	22/32	0/10	6/31	29/32	4/10	6/14	25/31	3/10
Liver	2/24	6/29	0/10	1/31	14/32	1/10	3/31	19/32	3/10	2/14	23/31	2/10
Kidneys	0/24	6/29	0/10	3/31	18/32	0/10	8/31	19/32	4/10	2/14	24/31	0/10
Heart blood	0/24	1/29	0/10	0/31	6/32	0/10	0/31	12/32	0/10	2/14	11/31	2/10

*) Numerator = No. of positive findings; Denominator = No. mice pairs.
**) For designation of the series, see Table 2.

A second series of experiments (11 tests) was made with attenuated and vaccinal anthrax strains. Among the latter were investigated the strains STI-1, Shuia-15, TSenkovskii-2. The mice were infected with suspensions of 5-24 hour old cultures with a centration of 5 x 10^8 organisms. As a result it was established that an inconstant ability of incapsulation during the first hours after inoculation was possessed only by the TSenkovskii strain No. 2; the other two vaccinal strains formed no capsules either in the inoculated or the control animals (which succumbed after 72-96 hours or were killed on the 10th day).

In order to elucidate the practical importance of a possible antagonism of the anthrax bacillus introduced in a mixture with other soil aerobes a series of 11 tests was made with mixtures of virulent anthrax bacilli and of saprophytes. These tests were made with 24 hour old cutlures; the proportion of the saprophytes in relation to the anthrax bacilli was 1:1 or 3:1. The doses consisted of 5 x 10^8 to 10 x 10^8 organisms. Thirty minutes after intraperitoneal administration of the mixtures one observed in 4 out of the 7 pairs of mice examined incapsulated anthrax bacilli while in 3 pairs uncapsulated organisms were present. One hour after inoculation in 9 out of 11 cases anthrax bacilli surrounded by capsules were observed and after two hours such organisms were found in all 11 cases. In 10 out of the 11 tests the pairs of control mice succumbed after intervals ranging from 20 to 168 hours. It is of interest to note that in one test (No. 64/59) the pair of control mice was killed on the 10th day and that it was impossible to obtain any cultures of bacilli from these animals.

In the test mice this group incapsulated anthrax bacilli were found already 30 minutes after infection and again after 60 and 120 minutes.

(b) Tests with inocula prepared with an adjuvant

In Table 4 are assembled the results of tests with virulent anthrax bacilli administered in suspensions made with an adjuvant.

Table 4

Findings of Incapsulated Anthrax Bacilli at Various Time Intervals after Infection of White Mice (see Table 2)

Number of Strains Examined	Suspensions Used		Times of Administration (Minutes)						
	Age	Concentration	60	120	180	240	300	360	60-360
15	24 hrs.	$5x10^6$-10^8	0/24	13/24	16/22	12/12	10/12	10/11	23/24

Time of the death of the control animals:
M ± m = 25.3 ± 0.86; sigma = ± 5.7

As proved by bacterioscopic examination, the incapsulated anthrax bacilli showed larger dimensions and bulkier capsules than those observed in the tests in which the organisms had been administered in normal saline. Still, as shown by Table 4, in comparison with the tests in which no adjuvant had been used, the onset of the capsule formation was delayed. Thus in the tests with the adjuvant 100% of capsule formation became manifest only 4 hours after inoculation, whereas in the case of analogous inocula suspended in normal saline (see Table 2, series 1) 100% of capsule formations by virulent anthrax bacilli were observed already 2 hours after infection.

A second series consisting of 6 tests, in which the mice were infected with 24 hour old cultures in amounts of 500 million of saprophytes and of the vaccinal strains STI-1 and TSenkovskii No. 2, administered in an adjuvant, showed, when the animals were killed after the time intervals indicated in Table 4, with the exception of the TSenkovskii strain the presence of uncapsulated gram-positive organisms. The adjuvant did not increase the virulence of the soil saprophytes, since in 3 out of 4 tests with these organisms the control mice survived throughout the observation period of 10 days; only in one test (with the B. anthracoides strain No. 23) the control mice succumbed 24 and 48 hours after infection, showing the presence of uncapsulated gram-positive organisms. Two pairs of mice infected with the vaccinal strains also succumbed 24 and 48 hours after infection; in the case of the animals infected with the TSenkovskii strain No. 2 incapsulated bacilli were observed, while in those infected with the STI-1 vaccine uncapsulated organisms were found. In the remaining 6 control mice, infected with saprophytes, which survived and were killed on the 10th day, bacilli were absent altogether.

Evaluation of the Results

The investigations made allow characterization of the accelerated identification of pathogenic anthrax bacilli with the aid of the biological test for incapsulation (5) as fully reliable, practically simple and epidemiologically (epizootologically) justified.

The use of this test enables the investigators to avoid factors not rarely met with in the case of the classical biological tests (carried on until the death of the animals) which may lead to misinterpretations (slightly marked morbid findings, anaerobic infections, microbial antagonism, lysis of capsular substance in the carcasses), not to speak of the long time required for the performance of the tests.

We established that for the accelerated biological test for capsule formation it is most rational to use young (3-5 hour old) cultures or even spores; this renders it practically possible to identify the anthrax bacilli already 3-5 hours after infection of the animals. Actually intraperitoneal administration of suspensions of the suspect material (4) to white mice permits to give a final decision after 90 minutes. Using our method in medical practice, A. Vita (14) recorded convincing results 7 hours after the examination of material from patients had been started.

Though the use of an adjuvant improves the capsule formation by virulent bacilli, this method had not been found reasonable in work with pure cultures, since in non-immune animals capsule formation takes place well and rapidly without an adjuvant; the time of capsule formation is briefer in this case.

The proposed method is suitable also in so far as, in contrast to other "express" bacteriological methods for the identification of anthrax bacilli, it permits at the same time to form an opinion in regard to the pathogenicity (virulence) of the organisms under test for the species of animals used; this is of great practical importance.

The sensitivity of the proposed method is not lower than that of the earlier methods for the diagnosis and demonstration of the anthrax bacillus described in the literature, this organism being characterized by infective doses of 10,000 spores (2) or 50,000 - 5,000,000 bacilli per ml of the suspensions (6).

Our experiences indicate that the variant of the proposed method (intermittent puncture of the peritoneal cavity) is rather unsuitable if white mice are used but suitable if use is made of larger animals - (non-immune) guinea pigs. Still, an excessively large number of punctures (more than 3) exerts an untoward influence also in this species of animals. Nevertheless one must not conclude that it is not advisable to use this variant of the accelerated test for capsule formation in guinea pigs. In practical work it is not necessary to make a large number of punctures in the guinea pigs as was done in our experiments. It is sufficient to make the first diagnostic puncture 3 hours after the inoculation. Only in the case of a negative result is it necessary to repeat the puncture after 1-1.5 hours.

A definite drawback of this method is that the diagnostic punctures of the peritoneal cavity of the infected guinea pigs are made "in the dark". This can be the source of methodical failures. Such failures naturally are fully absent if the animals are killed seriatim and this procedure is followed by autopsies and collection of the material under visual control. This method can be used most rationally in white mice.

There exist possibilities for modifications of this method, in this first line in regard to a restriction of the number of the animals (3 instead of 8-10) for the sake of economy and in regard to the optimal intervals of time for killing the animals after infection. Still, considering the various modifications one must realize that the reliability of a rapid result stands in direct relation to the number of animals tested and the intervals of time at which they are killed seriatim. The same holds true of the selection of the organs and tissues used for bacteriological examination.

Conclusions

1. The rapid identification of pathogenic anthrax bacilli with the aid of the biological test for capsule formation permits a laboratory diagnosis of anthrax during the first hours after the moment at which the material becomes available.

2. In order to enhance the reliability of the results of this method it is indicated to use a complex of bacteriological examinations of the tissues and organs of the killed animals - the exudate of the peritoneal cavity, the spleen, liver and kidneys.

3. It is rational to use for the accelerated biological tests spores or young (3-5 hour old) cultures suspected to be such of B. anthracis. It is also permissible to make the intrapertioneal inoculations directly with suspensions of the pathological material.

4. The proposed method can be used by any medical or veterinary bacteriological laboratory.

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(#97)

IDENTIFICATION AND DETERMINATION OF THE PATHOGENIC PROPERTIES OF THE ANTHRAX BACILLUS WITH THE AID OF THE BIOLOGICAL TEST FOR INCAPSULATION

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(Original pp. 150-151)

In 1960 there appeared in the medical literature a proposal by E. N. Shliakhov and E. V. Gruz for a new biological method for the rapid identification and determination of the anthrax bacillus (1,2).

The principle of the accelerated biological test was to administer to white mice intraperitoneally a suspension of the suspect bacilli and to kill the animals seriatim after 30, 60, 120 and 180 minutes. Identification was achieved with the aid of a microscopical examination of smears of the peritoneal exudate or of other organs of the mice (incapsulated gram-positive bacilli).

In its original form the method of Shliakhov and Gruz envisaged the use of 8-10 infected white mice, out of which 3-4 pairs were killed after 30-180 minutes, while 2 mice were kept as controls to their natural death or to observe their survival during a period of 10 days.

In our laboratory we decided to kill the inoculated mice seriatim only after two intervals, after 90 and 120 minutes, using not pairs but single mice for each test and to keep a third mouse under observation until the end of the test.

We prepared the infective suspensions from pathological material from a sick (succumbed) animal- from the spleen, lymph nodes, ear - through grinding the material in normal saline. The suspension was administered in an amount of 0.5 ml intraperitoneally to two white mice and subcutaneously to a third (classical biological tests as control) which remained under observation.

The first two mice were killed after 90 and 120 minutes. Smears of the peritoneal exudate were stained according to Mikhin's method and were microscopically examined to detect incapsulated anthrax bacilli.

In 1961-1962 we made 5 investigations with the aid of the method of Shliakhov and Gruz:

1. In June 1961 we received the spleen and lymph nodes of a calf privately owned by an inhabitant of Berdichev. The pathological material became available on the day the calf had been killed.

2. In July of the same year we received the spleen and lymph nodes of a calf belonging to an animal-breeding farm infected with anthrax. This material came to hand on the 2nd day after killing of the calf.

3. In October 1961 were received the partly decomposed spleen and lymph nodes of a privately owned cow which had been subjected to emergency killing (Notschlachtung).

4. In the same month were received the ear and a piece of the spleen of a cow from a kolkhoz herd which had died suddenly. The material was received one day later.

5. In August 1962 came to hand the spleen of a cow belonging to a private person which had been subjected to emergency killing. A suspension made from this organ was administered to 2 mice (to one intraperitoneally, to the other subcutaneously). The first mouse was killed after 120 minutes, the other was kept until its death.

After the mice had been killed seriatim (after 90 and 120 minutes) in all cases anthrax bacilli surrounded by capsules were observed in the peritoneal cavity. Cultivations made with the peritoneal exudate and the heart blood in MPB and on MPA yielded after one day pure cultures of B. anthracis.

The subcutaneously infected mice succumbed in all cases to anthrax sepsis within 12-24 hours or after longer periods.

The above recorded observations led to the following conclusions:

1. With the aid of the biological test of Shliakhov and Gruz it is possible to identify the anthrax bacilli 1.5 to 2 hours after inoculation of the pathological material while the animals tested according to the classical method succumb after 12-24 hours or after longer periods.

2. The proposed method is more sensitive and rapid than the classical method but requires a somewhat larger number of test animals (8-10); however, it is sometimes possible to use fewer animals.

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(#98)

TESTS WITH THE SPECIFIC BACTERIOPHAGE "BA-9" FOR THE IDENTIFICATION OF ANTHRAX BACILLI

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(Original pp. 152-156)

In laboratory practice not rarely the methods used for the identification of the isolated microbial cultures are quite unwieldy and require a considerable time for their application. Among the large number of methods for the bacteriological diagnosis and identification of microbial cultures the use of bacteriophages is considered as one of the most specific, rapid and simple methods (2, 3, 4, 5, 14, 15). This method is of particular value for the recognition of so-called "atypical" cultures. Apparently this holds true also for the identification of the anthrax bacillus with the aid of a specific bacteriophage.

As stated in the literature, Soviet and foreign observers (Monteiro, 23; F. N. Rozgon, 11; Cowles and Hale, 24; F. A. Terent'ev, 12; A. P. Zotov, 7, 8 and others) isolated strains of anthrax bacteriophages from various extrinsic substrates (water, soil, old anthrax cultures, etc.).

Still, these bacteriophages were not used for the identification of anthrax cultures and only in 1951 McCloy (22) reported on the isolation, from an atypical B. cereus strain, of a highly specific strain of anthrax bacteriophages ("W") which lysed all 171 cultures of B. anthracis tested but only 2 out of 56 cultures of B. cereus.

Further isolations of anthrax bacteriophages and diagnostic use of them were made by Brown and Cherry (17); Brown and Moody (16); Ivanovics and Lantos (18); Stamatin (26); Stamatin and Anghelesco (27); Leise et al. (21); Seidel (25) and others.

In the present paper data are furnished on the isolation and investigation of a specific anthrax bacteriophage, conditionally named BA-9, and also data on parallel tests with this phage and the phages "E" of Stamatin, "alpha-g" of Ivanovics and Lantos and "W" of McCloy.

Materials and Methods

1. Objects for the isolation of phages were soil samples, collected at various points of Kishenev.

2. Used were the following nutrient media: 1.5% and 0.7% meat-peptone agar and meat-peptone broth. The pH of the media was 7.2-7.4.

3. As test cultures were used the strains 1938 T (asporogenic, received from Stamatin) and "Davis" (asporogenic).

4. Specificity tests were made with the following phages: "BA-9", isolated in our laboratory in 1962; "E" - isolated in 1957 from the soil of Bucharest by Stamatin; "alpha-g", obtained from Ivanovics and "W", isolated by McCloy.

5. The lysogenicity was studied with the following 95 strains of aerobes:

a) B. anthracis	52 strains	f) B. mycoides	2 strains
b) B. cereus	30 -"-	g) B. mesentericus vulgatus	1 strain
c) B. subtilis	3 -"-	h) B. brevis	1 -"-
d) B. anthracoides	4 -"-	i) B. megatherium	1 -"-
e) B. pseudoanthracis	1 strain		

According to their origin the strains fell into the following categories:

Table 1

Number of Strains	Isolated from: From Man	From Cattle	From "Small" Cattle*	From Horses	From Pigs	From the Soil of a Cattle Grave
52	15	4	2	1	1	5

From Imported Raw Materials	Unknown	Vaccinal Strains
12	7	5

*) I. e., sheep and goats.

6. Production of the bacteriophage "BA-9": A quantity of 2.5 g of soil was mixed with 50 ml of meat-peptone broth, ground up in a mortar and the mixture was transferred into sterile flasks. Added were 2 ml of an 18-20 hour old culture of an indicator strain grown in broth or on agar (in this case suspensions were made the concentration of which did not exceed that of a 20 hour old broth culture). The flasks were incubated at 37°C for 48-72 hours; then the supernatant fluid was passed through Seitz filters with No. 3 filter membranes. A drop of the filtrate was put on the surface of an 1.5% meat-peptone agar plate, inoculated with the indicator culture. Before transfer of the filtrate the indicator culture plates were dried for 1 hour with open covers.

In the case of the presence of a specific phage in the filtrate one could observe either complete lysis or spotwise lysis in the tracks of the filtrate drop.

7. Determination of the phage titer: Determinations of the phage titer were made according to the method of Appelman or that of Gratia. Concentrations of the phages were made with the aid of passages of the phages and the indicator cultures on MPA (meat-peptone agar).

8. Tests for the specificity of the phages: Into Petri dishes with a quite even bottom one poured melted 1.5% MPA. After cooling of the agar the bottom of the plates was marked in squares with a side length of not less than 2 cm. The plates were dried in a thermostate and then with the aid of a loop with a diameter of 5 mm one put in each square one drop of a 5-6 hour old growth of the culture tested. The plates were dried once more in the thermostate with open covers for 30 minutes; then with the aid of a loop with a diameter of 2 mm drops of the bacteriophage were put on the center of the dried drops of the culture. After 8-10 hours' incubation the results (presence and degree of lysis) were read.

Results of the Investigations

With the aid of the above mentioned method we examined 11 samples of soils. The filtrates of two of them (Nos. 7 and 9) exerted a lytic action, but a further passage of the lytic agent in sample No. 7 proved impossible.

The filtrates of sample No. 9 showed in the first passages on the indicator culture evenly distributed lytic spots with a diameter of 3 mm with an even outline.

Through further 15 passages in MPB the titer of the bacteriophage became increased to 10^{-8} and complete lysis was noted round the drops of the phage.

The product was tentatively called the phage "BA-9". Its specificity was tested on 52 anthrax strains with a varying virulence and 43 strains of various spore-bearing saprophytes. As a result of the specificity tests with the phage "BA-9" in the initial investigations one anthrax strain (No. 64) produced no lysis and the 43 strains of saprophytes did not become lysed with the exception of the B. anthracoides strain No. 86, which showed some impairment of the growth at the site of administration of the phage.

Further investigations of the anthrax strain No. 64 showed that it was contaminated; after it had been rendered pure, it became lysed by the phage "BA-9".

Besides those made with the phage isolated in our laboratory, specificity tests were made with the phages "E", "alpha-g" and "W". As a result it was established that all 52 anthrax strains were lysed by these three phages.

Using the phage "E" for tests on saprophytes one noted some impairment of growth in the case of the B. anthracoides strain No. 76 and lysis of a single colony of the B. anthracoides strain No. 22. The phage "alpha-g" also produced lysis of single colonies of these strains.

The results of the specificity tests with the phages are shown in Table 2.

The strains No. 76 and 22, showing some impairment of the growth at the site of administration of the phages, according to other characteristics (MPB, MPA, motility, hemolysis, "pearl string" test, etc.) had to be classified as anthrax-like strains; the character of their lysis differed markedly from that shown by anthrax cultures.

Table 2

Results of the Action of Anthrax Phages on the Cultures of Different Bacilli

Kind of Strains	No. of Strains Examined	Phage "Ba-9" Positive	Phage "Ba-9" Negative	Phage "E" Positive	Phage "E" Negative
(1) B. anthracis	52	52	-	52	-
(2) B. cereus	30	-	30	-	30
(3) B. subtilis	3	-	3	-	3
(4) B. anthracoides	4	1 (±)	3	2 (±)	2
(5) B. pseudoanthracis	1	-	1	-	1
(6) B. mycoides	2	-	2	-	2
(7) B. mesentericus vulgatus	1	-	1	-	1
(8) B. brevis	1	-	1	-	1
(9) B. megatherium	1	-	1	-	1

Kind of Strains	No. of Strains Examined	Phage "alpha-g" Positive	Phage "alpha-g" Negative	Phage "W" Positive	Phage "W" Negative
(1)	52	52	-	52	-
(2)	30	-	30	-	30
(3)	3	-	3	-	3
(4)	4	2 (±)	2	-	4
(5)	1	-	1	-	1
(6)	2	-	2	-	2
(7)	1	-	1	-	1
(8)	1	-	1	-	1
(9)	1	-	1	-	1

Thus the results obtained indicate a high specificity of the anthrax bacteriophage "BA-9" isolated by us - the specificity being only slightly below that of the phages "E", "alpha-g" and "W". The results of the test can be read already after 8-10 hours.

With the aid of this method it is possible to test all suspicious anthrax colonies. The method of phage-diagnosis is suitable for the identification of virulent and avirulent strains of B. anthracis, in the case of which latter pathogenicity tests are unsuitable.

Conclusions

1. The bacteriophage "BA-9", isolated by us from soil, proved to be specific lytic agent for the 52 anthrax strains tested.

2. The same high specificity was shown by the phages "E", "alpha-g" and "W".

3. The anthrax bacteriophages studied can be used in the complex of tests for the laboratory diagnosis of B. anthracis.

4. Phage-diagnosis can be recommended as a rapid method for the identification of microorganisms.

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(#99)

DIFFUSIVE PRECIPITATION AS A METHOD FOR THE DETECTION OF THE ANTHRAX ANTIGEN

(A Preliminary Communication)

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(Original pp. 157-163)

The method of diffusive gel precipitation has won wide recognition within recent years inasmuch it is one of the most specific method for the immuno-chemical analysis of complex antigens. With its help one may detect the presence of separate antigens in the complexes studied. In contrast to the precipitation reaction in the liquid phase, where the lines of precipitation become joined in one ring, in the case of the diffusive precipitation, owing to the different rapidity of the diffusion, lines of precipitation strictly corresponding to the pairs of antigens and antibodies form in a quantitative relation to the presence of the antigens at definite distances from each other (1-5).

As is known, the precipitation reaction was recommended for the detection of the anthrax antigen in the substrates in 1910 by Ascoli and Valenti and this method is used successfully mainly in veterinary practice. According to the classical method the antigen for Ascoli's reaction is obtained with the aid of thermo-extraction; it must be transparent.

For medical practice Ascoli's reaction has been recommended by Stanishevskaia and Burgos (6). Many years later this method was again proposed by Cornelson et al. (7) and, according to some statements (8), it gives up to 85% positive results with the scabs of the patients. According to the same statements at the same time the frequency with which anthrax bacilli are isolated from the site of the skin affections does not exceed 48% and at present, owing to the inhibitive therapy with antibiotics, this percentage is still lower.

The aim of the present paper was to test the various antigen-containing anthrax substrates with the aid of double diffusive gel precipitation in respect to the specificity and expediency of the method. Inasmuch as the physico-chemical basis of gel precipitation differs from that of Ascoli's reaction, we

found it possible to observe the precipitation phenomena taking place as a result of the diffusion of dissolved antigens during the process of growth of the microbial population, i.e. without any supplementary treatment of the antigen-containing substrates. As such substrates served the scabs of patients and the bacillary form and spores of B. anthracis and the related saprophytes.

Materials and Methods

Agar gel. From Far-Eastern agar--agar which had been washed in running water for some days and then dried--was prepared a 1.5% gel in a 0.075 M phosphate buffer with a pH of 7.3 The agar was clarified with egg white, filtered, distributed in flasks and after autoclaving at the one atmosphere for 20-30 minutes was kept in the refrigerator.

Agar with the addition of nutrient serum was prepared immediately before use through the addition under sterile precautions to agar which had been melted and cooled to 50°C of 10% normal horse serum or fresh inactivated serum of sheep blood.

Preparation of agar gel plates. Agar which had been melted and cooled to 50°C was poured into Petri dishes in layers of 2.5-3mm. In the agar layer holes were made with a diameter of 6 mm and 6 mm distant from one another. A thin layer of melted agar was placed into the bottom of the holes. The latter were arranged in 3 or 5 rows or in a circle. Rows which had been filled one day earlier with precipitating serum alternated with such filled with antigen.

Precipitating serum. Use was made of the standard anthrax precipitating serum of the Orlov Biofabrika, series 91 and 85.

Production of adsorbed precipitating serum. To produce this serum 24 hour old growths on MPA slants, washed off from 5 tubes with a small amount of normal serum were centrifugated, twice washed in normal saline and 30 ml of the standard anthrax precipitating serum were added. Adsorption was made at room temperature for 5, 15, 30 and 60 minutes. The adsorbed serum was separated from the bacterial mass through centrifugation of 5,000 revolutions for 10 minutes. Sterilization was effected with the aid of passage through gradocol filters No. 3. As a result sera were obtained adsorbed separately with strains of B. anthracoides and B. pseudoanthracis and also such adsorbed with a mixture of these strains.

Antigen-containing Materials

1. Microbes:

a) Suspensions of bacilli in the vegetative form: 2 vaccinal strains (STI-1 of Ginsburg and a commercial strain of the TSenkovskii vaccine No. 2); 2 virulent anthrax strains; 3 B. anthracoides strains; 2 B. subtilis strains; 2 B. megatherium strains from our collection; 26 B. cereus strains isolated from soil and kindly put at our disposal by Professor Afrikanian (Erevan) and one strain each of B. pseudoanthracis and B. myccides.

b) Spore suspensions of 2 virulent B. anthracis strains; 3 B. cereus strains; 3 B. anthracoides strains; 1 B. subtilis strain and 1 B. pseudoanthracis strain.

2. Parts of the scabs taken from patients suffering from cutaneous form of anthrax.

3. Standard anthrax antigen:

Preparation of the microbial suspensions. 5 and 24 hours old growths of each of the cultures under test on MPA slants were washed off with small quantities of normal saline. The holes (in the agar plates) were filled with the suspensions to the top.

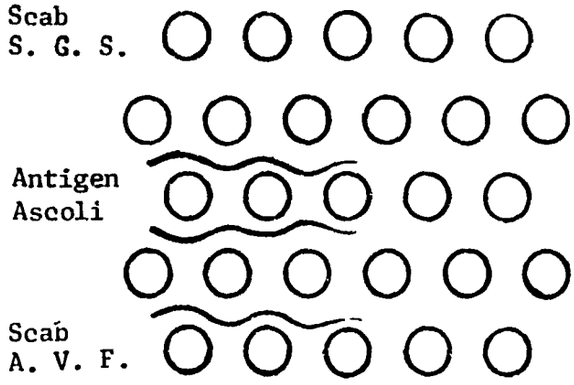
Preparation of spore suspensions. Cultures on agar slants (MPA of Hottinger) of each of the strains under test grown for 5 and 24 hours were kept at room temperature for 5 days; then the growths were washed off with distilled water into sterile test tubes and the suspensions were kept in the refrigerator for 5-6 days. Counts of the spores were made in the chamber of Goriaev with the aid of the phase contrast apparatus of the microscope MBI-1 with the objective 20x and the ocular 10x and the binoculars AU-12-1.5x. The concentration of the spores was determined according to the formula N= 250,000 pr, in which p stands for the amount of spores in one large square and r for the degree of dilution of the suspensions (9).

Preparation of extracts from the scabs. Particles of the scabs taken from the patients were fragmented, a 5-10 times larger amount of normal saline was poured over them and the fluid was kept in the refrigerator for 18-24 hours. An amount of 0.2 ml was required per test. A transparency of the extracts was not obligatory.

Performance of the Tests and Their Results

Study of materials from the scabs. The tests were made in Petri dishes with agar gel to which a 1:10 000 solution of sodium merthiolate had been added. The holes were arranged in rows. The material under test was put into the first hole of a row in an undiluted state and dilutions made respectively in proportions of 1:2, 1:4, 1:8 and 1:16 were put into the following holes. For the purposes of a positive control Ascoli's standard anthrax antigen was put into the holes of one row in the same proportions. Between the rows with the antigen was one row of holes with undiluted standard anthrax precipitating serum (Drawing 1). The dishes were kept at room temperature for 48 hours when readings were taken.

For the investigations scabs obtained from 10 patients were used. In 8 of them a clinical diagnosis of anthrax (cutaneous form) had been made; in one instance (patient T.A.A.) this diagnosis was doubtful and in the case of the patient TS. D.S. the preliminary diagnosis was "pneumococcal conjunctivitis".



Drawing 1. Diffusive gel precipitation with extracts from the scabs. Above - negative precipitation reaction; below - positive reaction with scab extract from patient A. V. F. In the In the center - positive control.

In all patients besides gel precipitations bacteriological examinations and biological tests on mice were made as well as intracutaneous allergic tests with chemical anthraxin. Results of the tests are shown in the following table:

Initials of the Patients	Results of Gel Precipitation Tests	Bacteriological Examinations	Allergic Tests
V. N. I.	+	-	+
A. V. F.	+	-	+
G. A. P.	-	-	+
L. D. P.	+	-	+
S. G. S.	-	-	+
TS. D. S.	+	-	+
K. E. G.	+	+	+
B. D. V.	+	+	+
V. G. P.	-	-	+
T. A. A.	-	-	-

In the case of the patient T.A.A. all laboratory tests gave a negative result. In conjuction with the unclear clinical findings this permitted the rejection of the diagnosis of anthrax.

Among the 9 patients with a final diagnosis of anthrax bacteriological investigations gave a positive result only in two instances. Gel precipitation tests made with the scabs and anthrax precipitating serum gave a positive results in 6 of the 9 patients, i.e. in 2/3 of the cases. It is characteristic that in all these cases allergic skin tests with chemical anthraxin also gave a positive result.

Thus positive results of gel precipitation tests with material from the scabs of the patients rendered it possible to confirm, hand in hand with the anthraxin tests, in a number of cases the diagnosis of anthrax and and to reject this diagnosis in one instance.

Tests with the Use of the Vegetative Forms of the Organisms as Source of the Soluble Antigen

The tests were made in dishes with agar gel to which nutrient serum had been added. The standard precipitating anthrax serum was used in undiluted form and in dilutions of 1:2, 1:4, 1:8 and 1:16.

After the antigen had been put into the holes, the plates were incubated either in the ordinary atmosphere or in an atmosphere containing 25% carbon dioxide. The presence of precipitation lines was studied after 6, 12, 18 and 24 hours; then, in order to prevent a further growth of the organisms, the plates were put into the refrigerator. Forty eight hours after incubation had been started a 1:10 000 solution of sodium merthiolate was poured into the dishes and left for 2 hours; then the growth was washed off and final readings of the precipitation lines were made. Further observations were made in the course of 10 days.

In the plates incubated for 6 and 12 hours with undiluted anthrax precipitating serum one could observe the appearance of one broad precipitation line in the case of both vaccinal strains as well in that of the anthrax strains, in 3 out of 26 B. cereus strains, in all investigated strains of B. anthracoides and B. pseudoanthracis.

In the case of the same strains one could observe in the plates incubated for 18 and 24 hours 2 more narrow precipitation lines situated nearer to the antigen. Sometimes only 1 or 2 precipitation lines were found.

The anthrax strains as well as the anthrax-like strains showed precipitation also when serum dilutions of 1:2 and 1:4 were used (1 or 2 lines).

Investigations of the Soluble Antigen in Spore Suspensions

The conditions of the tests were the same as those for the study of the vegetative forms. The antigen was put into the holes in the form of 0.05 ml of the spore suspensions of the strains under test, the concentrations of the suspensions equalling 100, 10,000 and 1 million spores per ml. Thus into each hole were put respectively 5,500 or 50,000 spores. The introduction of 5 spores proved insufficient - no lines of precipitation were seen.

All strains tested (2 anthrax strains, 3 B. anthracoides strains, 3 B. cereus strains and one strain each of B. subtilis and B. pseudoanthracis) in concentrations of 10,000 or 1 million spores per ml gave 1-3 precipitation lines with standard anthrax precipitating serum after incubation for 12, 18 and 24 hours.

Tests with Adsorbed Anthrax Precipitating Serum

The tests were made in the manner described above but in the place of standard anthrax precipitating serum adsorbed precipitating sera were used.

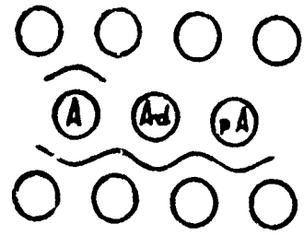
As antigen served 24 hour old agar cultures of 3 anthrax strains, 2 B. cereus strains, 2 B. anthracoides strains and 1 B. pseudoanthracis strain.

Each of these strains was tested with precipitating anthrax sera previously exhausted with one or two strains of anthracoids. In different tests better results were obtained with sera adsorbed for 15, 30 or 60 minutes. In these tests the sera, adsorption of which had been made under optimal conditions, showed a specific line of precipitation with B. anthracis and no precipitation with the strains used for adsorption (Drawing 2)*. In the other cases the strains produced precipitation with all strains or had been exhausted to such a degree that precipitation was absent in the case of all strains under test. In the cases in which a specific precipitation was obtained with B. anthracis alone, only one line of of precipitation was observed.

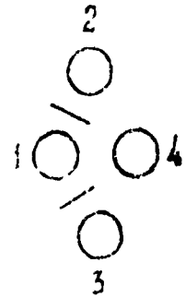
Evaluation

Tests made with vegetative forms and spores showed that the pathogenic strains of B. anthracis as well as the strains STI-1 and TSenkovskii-2 cause gel precipitation with the standard anthrax precipitating serum. The formation of several precipitation lines is related to the presence of several antigens appearing in the course of growth of the anthrax bacillus. Still, it was not possible to establish a regularity in the appearance of the precipitation lines since sometimes only 1 or 2 lines were formed.

Nevertheless it was possible to establish that firstly the intensity of the precipitation lines depends within certain limits upon the amount of spores used and secondly that, when vegetative forms are used, the precipitation takes place more rapidly than when spores serve as the antigen.



Drawing 2. Diffusive gel precipitation with adsorbed (upper row of holes) and not adsorbed (lower row) anthrax sera. A - antigen of B. anthracis; Ad - antigen of B. anthracoides; pA - antigen of B. pseudo-anthracis.



Drawing 3. Arrangement of the holes in the test. 1: Precipitating anthrax serum; 2: Ascoli's anthrax antigen; 3: Antigen under test; 4: Normal horse serum.

Some soil bacilli, like B. subtilis, B. mesentericus and B. megatherium produce no gel precipitation with anthrax precipitating serum. Of the 26 B. cereus tested only three produced precipitation. B. anthracoides (3 strains) and B. pseudoanthracis (2 strains) showed precipitation reactions analogous to those of the anthrax bacillus. Attempts to differentiate these bacilli according to the number or the character of the precipitation lines obtainable with various dilutions of the standard precipitating serum gave no positive results.

Still, the anthrax strains and the aerobes near to them in respect to their taxonomy, even though they have some common antigens, can be differentiated with the aid of precipitation tests made with an adsorbed precipitating serum in which the group antigens have been exhausted. Such adsorbed sera give precipitation only with B. anthracis. This method is suitable for the identification of anthrax bacilli in contaminated materials; still, in regard to its expediency and accuracy its value is considerably below that of the method of observing B. anthracis with the aid of adsorbed luminescent sera (10, 11).

Thus one may conclude that owing to the presence of features in the antigenic structure of B. anthracis common with those of some soil aerobes the method of gel precipitation with standard anthrax precipitating serum is of slight value for a detection of the anthrax bacillus in objects of the extrinsic environment.

Still, the method of gel precipitation with antigen from the scabs of patients even with standard anthrax precipitating serum can be considered as fully specific, since anthrax bacilli alone can be the source of the soluble antigens in the scabs. This reaction, side by side with Ascoli's reaction made with the scabs can be used quite legitimately for diagnostic purposes in medical practice - particularly if one considers the low percentage of a bacteriological confirmation of the diagnosis of anthrax and the fact that in the presence of negative bacteriological findings the precipitation reaction with the scabs often gives a positive result.

The method can be used easily in practical laboratories. The holes can be made with a corkscrew with an adequate diameter. The agar may be prepared with normal saline with addition of sodium methiolate and may be stored in stoppered containers for an unlimited time. The most reasonable position of the holes is

shown in Drawing No. 3. Here the standard anthrax antigen serves as control of the antigen and normal horse serum as control of the antibodies. In the case of positive reactions precipitation lines must form between the holes 1 and 3 and 1 and 2, and no lines ought to be present between the holes 2 and 4 and 3 and 4. The reactions ought to be read 50 hours after the holes have been filled.

Great attention should be paid to the fact that only 3 out of 26 B. cereus tested produced gel precipitation with anthrax serum as did all strains of B. pseudoanthracis and B. anthracoides. Since, as can be gathered from the literature, the criteria of a systematization of the variants of B. cereus are still under discussion (12), it is quite possible that the immuno-chemical relationship of single strains of this organism with pseudoanthrax and anthrax-like organisms can be used for for an elucidation of the taxonomy of the organisms.

Conclusions

1. In the process of growth the anthrax bacilli excrete into the surrounding medium soluble antigens, capable of becoming diffused in agar gels. These antigens may be detected in agar gels with the aid of precipitation tests with anthrax precipitating serum.

2. The standard anthrax precipitating serum also produces gel precipitation of the antigens of B. anthracoides and B. pseudoanthracis; as a consequence this serum is not suitable for the identification of the anthrax bacillus (antigen) in materials of the extrinsic environment.

3. Adsorbed precipitating anthrax serum may be used in gel precipitation tests for the identification of anthrax bacilli. However, in respect to its expediency and accuracy this method is of lesser value than that of tests in which adsorbed luminescent sera are used.

4. The gel precipitation reaction made with extracts of the scabs or other materials obtained from patients is specific and may be used in medical practice as a method for the laboratory diagnosis of anthrax.

5. An immuno-chemical study of certain strains of B. cereus, anthracoides and pseudoanthracis may be of preliminary value for a systematization of these organisms.

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13. ABSTRACT

This is a straight translation of the book on Anthrax by E. N. Shliakhov of the Moldavian Scientific Research Institute of Epidemiology, Microbiology and Hygiene. It contains several articles on various aspects of the Immunology, Clinique and Diagnosis of Anthrax under two main headings after a brief synopsis of recent progress in understanding the disease: Anthraxin and Its Reactions, and Methods of Laboratory Diagnosis and Identification of the Causative Organisms. The illustrations in the book are also included here.

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KEY WORDS	LINK A		LINK B		LINK C	
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